STUDIES ON THE METABOLISM OF FATTY ACIDS IN n-ALKANE-UTILIZABLE CANDIDA YEAST(Dissertation_全文)

AUTHOR(S):

Okada, Hirofumi

STUDIES ON THE METABOLISM OF FATTY ACIDS IN n-ALKANE-UTILIZABLE CANDIDA YEAST

1986

HIROFUMI OKADA

Kyoto University

STUDIES ON THE METABOLISM OF FATTY ACIDS IN *n*-ALKANE-UTILIZABLE *CANDIDA* YEAST

1986

HIROFUMI OKADA

Kyoto University

PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The studies collected here have been carried out under the direction of Professor emeritus Saburo Fukui and Professor Atsuo Tanaka in the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engneering, Kyoto University, during 1981 - 1986.

It is the author's great pleasure to express hearty gratitude to Professor emeritus Saburo Fukui and Professor Atsuo Tanaka for their continuous guidance and encouragement throughout this work.

The author is also grateful to Dr. Mitsuyoshi Ueda for his invaluable suggestions.

The author is particularly indebted to Messrs. Toshitsugu Matsuki, Masaaki Uchida, Kazunori Yamanoi, and Tadashi Morikawa for their helpful discussions and collaboration. Thanks are also due to all members in Professor Tanaka's laboratory for their constant interest in the course of this work.

Hirofumi Okada

Laboratory of Industrial Biochemistry

Department of Industrial Chemistry

Faculty of Engineering, Kyoto University

CONTENTS

INTRODUCTION

In recent years, progress of biotechnology, such as genetic engneering, bioreactor, cell technology, prompts us to try producing useful materials by microorganisms. Various microorganisms, namely, bacteria, yeasts and fungi, are well known to be able to assimilate aliphatic or aromatic hydrocarbons as the sole source of carbon and energy (1). Because shortage of food becomes a serious problem, single cell protein (microbial cells), especially that from hydrocarbons, has been recommended as a novel source of food. Simultaneously, new fermentation technology has been developed to produce various metabolites such as amino acids, organic acids, carbohydrates, nucleotides, vitamins and coenzymes, antibiotics and so on from cheap raw materials, for example, hydrocarbons, alcohols and biomass (2-4). In these organisms which produce such useful compounds, investigation of metabolic pathway of these substrates as well as physiology of the organisms is supposed to contribute not only to high producibility of metabolites but also to discovery of new metabolic pathways and regulation mechanisms in these organisms. In the

present work, the author has tried to obtain some basal biochemical information on n-alkane-utilizable Candida yeasts.

Peroxisomes

Peroxisomes (microbodies) are subcellular organelles, appearing in eukaryotic cells. The organelles are morphologically characterized by a single limited membrane, a fine granular matrix, and often a crystalline or tubular core, and are biochemically characterized by the presence therein of one or more hydrogen peroxide-producing oxidases and catalase (5-7). Rhodin first observed the organelles by electron microscopy in rat kidney in 1954 (8). The term "microbody" was used for these organelles. de Duve and his coworkers isolated the organelles from rat liver for the first time and named these organelles "peroxisomes" on the basis of their function (9). Recently, it has been clarified that the treatments with hypolipidemic drugs (10,11), such as clofibrate, a kind of plasticizer (di-2-ethylhexyl phthalate) (12) and high fat diets (13) cause peroxisomes in rat liver to proliferate. From this fact, the organelles have been shown to play an important role in

the degradation of fatty acids (14,15). Tolbert et al. have isolated similar organelles from green leaves which participate in photorespiration in cooperation with mitochondria and chloroplasts (16) and called them "leaf peroxisomes". Meanwhile, Beevers et al. have found microbodies in the germinating castor bean which contain fatty acid β-oxidation system and glyoxylate cycle enzymes. They, therefore, called these organelles glyoxysomes (17).

Yeast peroxisomes (microbodies) were first observed by Avers et al. in the cells of Saccharomyces cerevisiae grown on glucose (18,19). They have asserted that these organelles contain several enzymes including catalase (20). However, the metabolic function of the peroxisomes was not clarified because the number of peroxisomes in the yeast cells was too small. During studies on the physiology and metabolism of alkane-utilizing Candida yeasts, Professer Fukui's reseach group has found a large number of specific organelles in n-alkane-grown cells compared with glucose-grown cells (Fig. 1) (21,22). The appearance and development of the organelles are related to alkane assimilation, and accompanied by a significant increase in the activity of catalase, a marker enzyme of peroxisomes. Since these

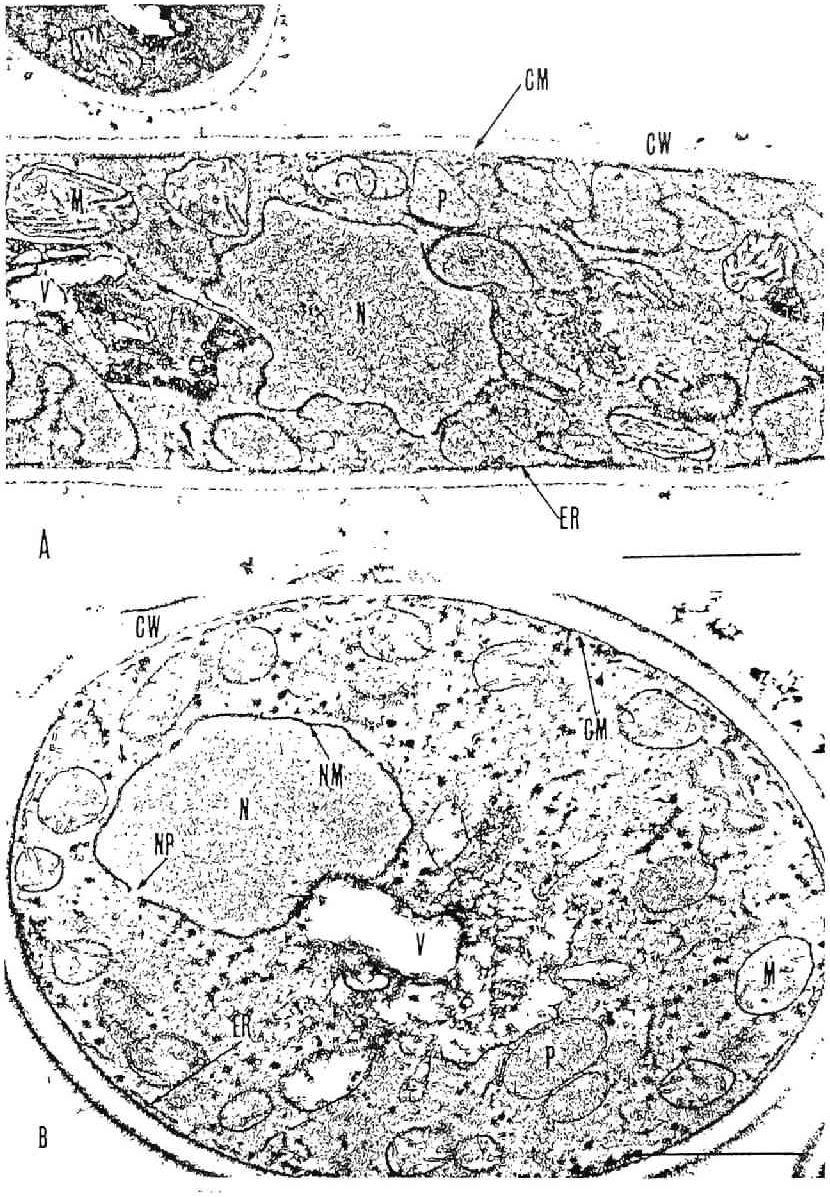


Fig. 1. Ultrastructure of Candida tropicalis pK 233 grown on alkanes (A) and on glucose (B).

CM, cell membrane; CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; NM, nuclear membrane; NP, nuclear pore; P, peroxisome; V, vacuole. Bar, 1 µm.

organelles can be stained cytochemically with 3,3'-diaminobenzidine, showing the localization of catalase, they should be designated as peroxisomes (microbodies). Also in methanol-utilizing yeasts, Professor Fukui's research group (23) as well as Europian groups (24,25) has demonstrated the presence of peroxisomes characterized by smaller number and larger in size than those of n-alkane-utilizing yeasts, and by the appearance of crystalline core. The organelles have been also found in algae (26), protozoa (27,28) and fungi (29), thus, they are shown to be in common in the eukalyotic cells.

n-Alkane assimilation pathway in Candida yeasts

The first step of alkane assimilation by yeasts is the uptake of exogeneous alkanes by cells. n-Alkanes incorporated into yeast cells are oxidized initially to the corresponding higher alcohols in microsomes. Although the catalytic mechanism of the hydroxylation system has not yet been elucidated, it is presumed to be composed of cytochrome P-450, NADPH-cytochrome c (cyt. P-450) reductase and a heat-stable lipid fraction (30). Higher alcohols are oxidized to the corresponding fatty acids via aldehydes. These

reactions are catalyzed by NAD-linked alcohol dehydrogenase and aldehyde dehydrogenase (31). Fatty acids are activated to the corresponding CoA esters by acyl-CoA synthetase (31) and degradated by the fatty acid β-oxidation system to yield acetyl-CoA (32) in peroxisomes, as the cases of castor bean (33) and rat liver (34). But it is not clear what enzymes participate in the fatty acid β-oxidation system of peroxisomes in the yeasts. Acetyl-CoA formed is further metabolized through tricarboxylic acid cycle (TCA cycle) which produces CO_2 and the reducing power linked to a respiratory system to yield energy, or glyoxylate cycle which plays an important role in the biosyntheses of cellular components including carbohydrates and amino acids. Glyoxylate cycle yields one molecule of C_4-compounds, such as malate and succinate, from two molecules of acetyl-CoA by condensation with oxalacetate and glyoxylate, respectively. Propionyl-CoA is also the final metabolite in the β-oxidation system when alkanes with odd carbon numbers are used as substrate. Propionyl-CoA formed is supposed to be metabolized through methylcitric acid cycle found in Candida lipolytica (35,36). In this cycle, propionyl-CoA is condensed with oxalacetate to form methylcitrate by

methylcitrate synthase, methylcitrate being converted to methylisocitrate, which is cleaved to succinate and pyruvate by methylisocitrate lyase. Then the succinate is metabolized *via* the TCA cycle. (Fig. 2)

When cultivated on alkanes, conspicuous numbers of peroxisomes appear in the yeast cells (21,22). This fact suggests that peroxisomes might play important roles in alkane assimilation. Professor Fukui's group has demonstrated that peroxisomes from the cells of alkane-grown *Candida tropicalis*, one of the typical alkane-utilizing yeasts, contain catalase, fatty acid β-oxidation system, part of glyoxylate cycle (isocitrate lyase and malate synthase) and so on (32,37). On the other hand, other glyoxylate cycle enzymes which are common to the TCA cycle, citrate synthase, aconitase and malate dehydrogenase are localized in mitochondria, but not in peroxisomes. These facts indicate that peroxisomes must cooperate with mitochondria in the metabolism of alkanes. Since fatty acid β-oxidation activity could not be detected in mitochondria (32), acetyl-CoA required for the citrate synthesis must be transported to mitochondria from peroxisomes. Carnitine acetyltransferase in peroxisomes and mitochondria might be

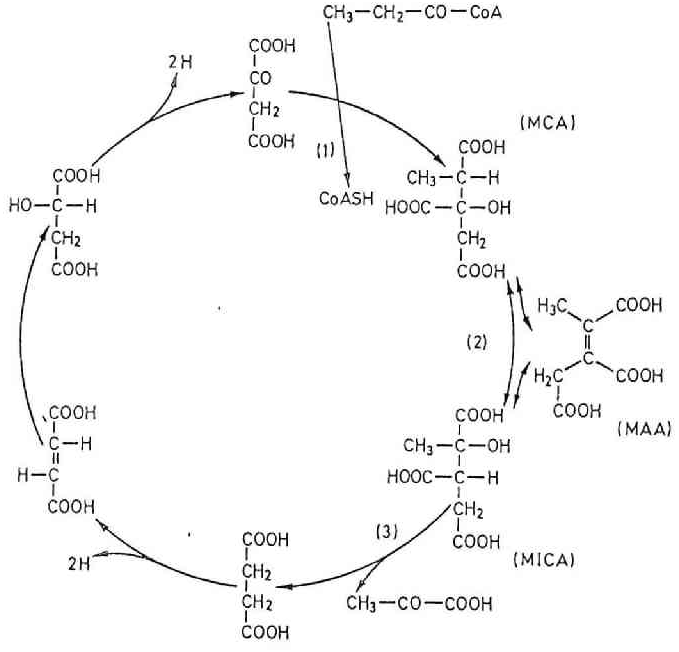


Fig. 2. Methylcitric acid cycle in C. lipolytica (35).

Abbreviations: MAA, methylaconitate; MCA, methylcitrate; MICA, methylisocitrate. Enzymes: 1, methylcitrate synthase; 2, aconitase; 3, methylisocitrate lyase

responsible for this transportation (38). Fig. 3 illustrates possible roles of microsomes, mitochondria and peroxisomes in alkane-utilizing C. tropicalis cells. Methylcitric acid cycle, a pathway of propionyl-CoA metabolism, seems to be located in mitochondria, since one of the key enzyme of the cycle, methylcitrate synthase, has been found in mitochondria of C. tropicalis cells grown on alkanes containing odd-chain ones (39).

Functions of TCA cycle and glyoxylate cycle

In TCA cycle, acetyl-CoA is condensed with oxalacetate to yield citrate by citrate synthase. Citrate is metabolized through TCA cycle to oxalacetate, where two molecules of CO_2 are released and reducing powers, which are used to generate ATP by oxidative phosphorylation connected to the respiratory chain, are provided. Thus, a complete turnover of the cycle results in the oxidation of C_2-unit from acetyl-CoA to CO_2. On the other hand, oxalacetate can be a precursor for gluconeogenesis where oxalacetate is converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase. Intermediates of TCA cycle are also used for the synthesis of amino acids. These facts indicate that

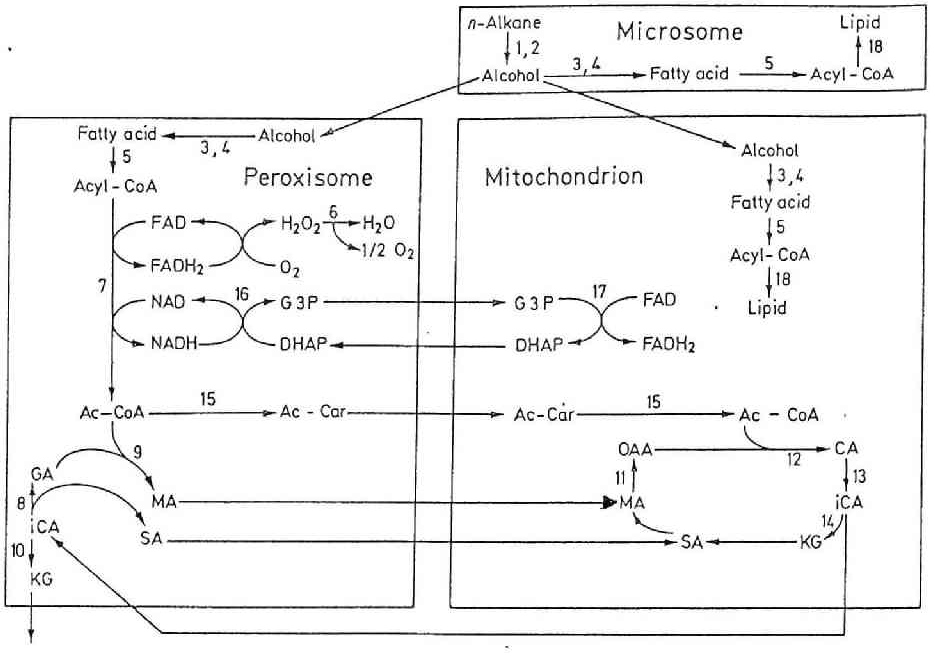


Fig. 3. Possible roles of peroxisomes in connection with those of mitochondria and microsomes in alkane assimilation by yeasts.

Abbreviations: Ac-CoA, acetyl-CoA; Ac-Car, acetylcarnitine; CA, citrate; DHAP, dihydroxyacetone phosphate; GA, glyoxylate; G3P, glycerol-3-phosphate; iCA, isocitrate; KG, α-ketoglutarate; MA, malate; OAA, oxalacetate; SA, succinate. Enzymes: 1, cytochrome P-450; 2, NADPH-cytochrome P-450 (cytochrome c) reductase; 3, alcohol dehydrogenase; 4, aldehyde dehydrogenase; 5, acyl-CoA synthetase; 6, catalase; 7, β-oxidation system; 8, isocitrate lyase; 9, malate synthase; 10, NADP-dependent isocitrate dehydrogenase; 11, malate dehydrogenase; 12, citrate synthase; 13, aconitase; 14, NAD-dependent isocitrate dehydrogenase; 15, carnitine acetyltransferase; 16, NAD-dependent glycerol-3-phosphate dehydrogenase; 17, FAD-dependent glycerol-3-phosphate dehydrogenase; 18, glycerophosphate acyltransferase

extra oxalacetate is necessary in TCA cycle to supply the intermediates for the syntheses of carbohydrates and amino acids. One possible pathway to produce oxalacetate is carboxylation of pyruvate by pyruvate carboxylase. An alternative pathway to synthesize oxalacetate is glyoxylate cycle. This cycle is composed of two key enzymes, isocitrate lyase and malate synthase, and the enzymes common to TCA cycle. Isocitrate lyase catalyzes the cleavage of isocitrate into succinate and glyoxylate. Malate synthase catalyzes the condensation of glyoxylate thus formed with acetyl-CoA to produce malate. Either of succinate or malate is metabolized via TCA cycle to yield isocitrate again. Thus, in glyoxylate cycle, the net synthesis of C_4-compounds is accomplished from two molecules of acetyl-CoA. This indicates that glyoxylate cycle is anaplerotic pathway to replenish C_4-compounds in C_2-compounds-utilizing microorganisms. (Fig. 4)

The glyoxylate cycle was first described in bacteria as the metabolic pathway for acetate or ethanol used as sole source of carbon (40). Later, it has become clear that this pathway has a larger representation in nature being operative under various nutritional conditions and physio-

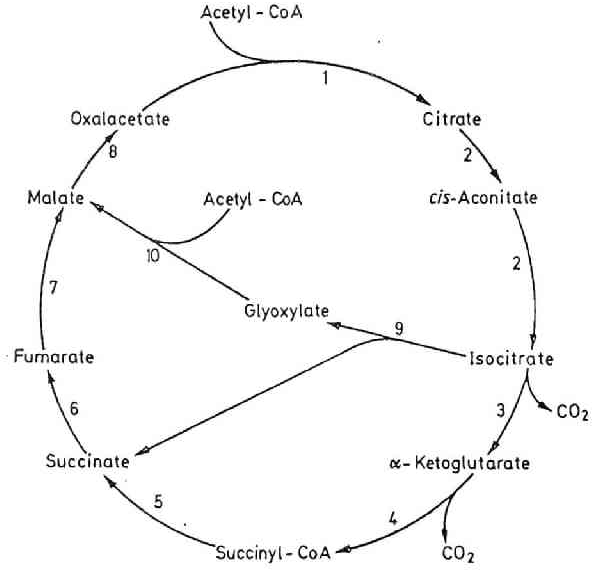


Fig. 4. TCA and glyoxylate cycles.

Enzymes: 1, citrate synthase; 2, aconitase; 3, isocitrate dehydrogenase; 4, α-ketoglutarate dehydrogenase; 5, succinyl-CoA synthetase; 6, succinate dehydrogenase; 7, fumarase; 8, malate dehydrogenase; 9, isocitrate lyase; 10, malate synthase

logical moments of the life of many organisms. In the germinating seeds of higher plants the cycle is important in the conversion of reserved lipids into carbohydrates (41). In algae the cycle participates in the utilization of acetate for growth when photosynthesis is not operative and carbohydrates are not available (42). In a certain nematodes, also, it seems to function in the convertion of reserved lipids to carbohydrates during embryogenesis (43). In yeasts, Duntze et al. first demonstrated the inducible nature of the key enzymes of glyoxylate cycle and observed that the synthesis of isocitrate lyase and malate synthase was regulated in response to the kinds of carbon source in the growth medium (44). Professor Fukui's group has showed that in a n-alkane-utilizable yeast, Candida tropicalis, the activities of isocitrate lyase and malate synthase are higher in the cells grown on n-alkane or acetate than in those grown on glucose and that glyoxylate cycle plays an indispensable and important role in the metabolism of n-alkane as well as acetate (45). However, molecular properties of these enzymes of the yeast are not investigated in detail.

Peroxisome biogenesis

The classical model for the biogenesis of peroxisomes is budding from the endoplasmic reticulum (ER) (46,47). This concept was based in part on morphological observations suggestive of connections between peroxisomes and ER, and in part on a biochemical study claiming that newly synthesized catalase was found in microsomes (48). Recent studies on the biosynthesis of peroxisomal and glyoxysomal proteins do not support this model. In vivo and in vitro experiments have demonstrated that rat liver catalase (49), Neurospora isocitrate lyase (50), cucumber cotyledon isocitrate lyase and malate synthase (51) are synthesized on free polysomes without detectable "pre-" or "pro-" sequences, released in cytoplasm and transported into peroxisomes. From these findings, Lazarow and de Duve have proposed a hypothesis that peroxisomal proteins enter the pre-existing peroxisomes by post-translational uptake (52). The mechanism of the transportation of peroxisomal proteins is similar to that of mitochondrial proteins (53), although mitochondrial proteins are synthesized as precursors with leader sequences, larger than mature proteins. On the contrary, most of peroxisomal proteins synthesized in

vitro are the same size as the corresponding mature proteins including catalase of Candida tropicalis (54) with exceptions of rat liver 3-ketoacyl-CoA thiolase (55), watermelon malate dehydrogenase (56) and larger subunit of Candida tropicalis carnitine acetyltransferase (57).

The development and maturing of peroxisomes is thought to be connected with the synthesis and transport of peroxisomal proteins. In this regard, the yeast cells are an excellent tool for studying the mechanism of biogenesis of peroxisomes and the transportation of peroxisomal proteins, because the development of peroxisomes can be easily controlled by changing the carbon source in the medium. In the studies of peroxisome biogenesis, purification of intraperoxisomal proteins and estimation of their molecular weights are important. However, peroxisomal enzymes of C. tropicalis purified hitherto, are only a few: Acyl-CoA oxidase, the enzyme participating in the initial step of peroxisomal β-oxidation system (58-60); catalase (61); and carnitine acetyltransferase (39,62). More peroxisomal enzymes should be purified and their properties have to be compared with those of the cytosolic counterparts for the detailed study of peroxisome biogenesis.

REFERENCES

(1) Fukui,S., Tanaka,A., Fujii,K., Shimizu,S., Inagawa,M. & Teranishi,Y. (1970) Kagaku 25, 638.

(2) Abbott,B.J. & Gledhill,W.E. (1971) Adv. Appl. Microbiol. 14, 249.

(3) Yamada,K. (1977) Biotech. Bioeng. 19, 1563.

(4) Fukui,S. & Tanaka,A. (1980) Adv. Biochem. Eng. 17, 1.

(5) Beevers,H. (1979) Ann. Rev. Plant Physiol. 30, 159.

(6) Masters,C.J. & Holmes,R.S. (1977) Physiol. Rev. 46, 323.

(7) Tolbert,N.E. (1981) Ann. Rev. Biochem. 50, 133.

(8) de Duve,C. & Baudhuin,P. (1966) Physiol. Rev. 46, 323.

(9) Baudhuin,P., Beaufay,H. & de Duve,C. (1965) J. Cell Biol. 26, 219.

(10) Lazarow,P.B. & de Duve,C. (1976) Proc. Natl. Acad. Sci. USA 73, 2043.

(11) Reddy,J.K. & Krishnakantha,T.P. (1975) Science 190, 787.

(12) Osumi,T. & Hashimoto,T. (1978) J. Biochem. 83, 1361.

(13) Ishii,H., Fukumori,N., Horie,S. & Suga,T. (1980)

Biochim. Biophys. Acta 617, 1.

(14) Masters,C.J. & Holmes,R.S. (1979) Trends Biochem. Sci. 4, 233.

(15) Lazarow,P.B. (1978) J. Biol. Chem. 253, 1522.

(16) Tolbert,N.E., Oeser,A., Kisaki,T., Hageman,R.H. & Yamazaki,R.K. (1968) J. Biol. Chem. 243, 5179.

(17) Briedenbach,W. & Beevers,H (1967) Biochem. Biophys. Res. Commun. 27, 462.

(18) Avers,C.J. & Federman,M. (1968) J. Cell Biol. 37, 555.

(19) Avers,C.J. (1971) Sub-Cell. Biochem. 1, 25.

(20) Perlman,P.S. & Mahler,H.R. (1970) Arch. Biochem. Biophs. 136, 245.

(21) Osumi,M., Miwa,N., Teranishi,Y., Tanaka,A. & Fukui,S. (1974) Arch. Microbiol. 99, 181.

(22) Osumi,M., Fukuzumi,F., Teranishi,Y., Tanaka,A. & Fukui,S. (1975) Arch. Microbiol. 103, 1.

(23) Fukui,s., Tanaka,A., Kawamoto,S., Yasuhara,S., Teranishi,Y. & Osumi,M. (1975) J. Bacteriol. 123, 317.

(24) Sahm,H., Roggenkanp,R., Wagner,F. & Hinkelmann,W. (1975) J. Gen. Microbiol. 88, 218.

(25) van Dijken,J.P., Veenhuis,M., Kreger-van Rij,N.J.W. & Harder,W. (1975) Arch. Microbiol. 102, 41.

(26) Collins,N. & Merrett,M.J. (1975) Plant Physiol. 55, 1018.

(27) Hogg,J.F. (1969) Ann. N. Y. Acad. Sci. 168, 281.

(28) Muller,M. (1975) Ann. Rev. Microbiol. 29, 467.

(29) Parish,R.W. (1975) Eur. J. Biochem. 58, 523.

(30) Duppel,W., Lebeault,J.M. & Coon,M.J. (1973) Eur. J. Biochem. 36, 583.

(31) Yamada,T., Nawa,H., Kawamoto,S., Tanaka,A. & Fukui,S. (1980) Arch. Microbiol. 128, 145.

(32) Kawamoto,S., Nozaki,C., Tanaka,A. & Fukui,S. (1978) Eur. J. Biochem. 83, 609.

(33) Cooper,T.G. & Beevers,H. (1969) J. Biol. Chem. 244, 3514.

(34) Lazarow,P.B. & de Duve,C. (1976) Proc. Natl. Acad. Sci. USA 73, 2043.

(35) Tabuchi,T. & Uchiyama,H. (1975) Agric. Biol. Chem. 39, 2035.

(36) Uchiyama,H., Ando,M., Toyonaka,Y. & Tabuchi,T. (1982) Eur. J. Biochem. 125, 523.

(37) Kawamoto,S., Tanaka,A., Yamamura,M., Teranishi,Y.,

Fukui,S. & Osumi,M (1977) Arch. Microbiol. 112, 1.

(38) Kawamoto,S., Ueda,M., Nozaki,C., Yamamura,M., Tanaka,A. & Fukui,S. (1978) FEBS Lett. 96, 37.

(39) Ueda,M., Tanaka,A. & Fukui,S. (1984) Eur. J. Biochem. 124, 205.

(40) Goldman,D.S. & Wagner,M.J. (1962) Biochim. Biophys. Acta 65, 297.

(41) Cooper,T.G. & Beevers,H. (1969) J. Biol. Chem. 244, 3507.

(42) Syrett,P.J., Merrett,M.J. & Bocks,S.M. (1963) J. Exp. Bot. 14, 249.

(43) Barrett,J., Ward,C.W. & Fairbairn,D. (1970) Comp. Biochem. Physiol. 35, 577.

(44) Duntze,W., Newman,D., Gancedo,J.M., Atzpodien,W. & Holzer,H. (1969) Eur. J. Biochem. 10, 83.

(45) Nabeshima,S., Tanaka,A. & Fukui,S. (1977) Agric. Biol. Chem. 41, 275.

(46) Hruban,Z., Swift,H. & Wisser,R.W. (1963) J. Ultrastruct. Res. 8, 236.

(47) Novikoff,A.B. & Shin,W.Y. (1964) J. Microscopie. 3, 187.

(48) Higashi,T. & Peters,Jr.,T. (1963) J. Biol. Chem. 238,

3952.

(49) Lazarow,P.B. (1980) Ann. N. Y. Acad. Sci. 343, 293.

(50) Desel,H., Zimmermann,R., Janes,M., Miller,F. & Neupert,W. (1982) Ann. N. Y. Acad. Sci. 386, 377.

(51) Kindl,H. (1982) Ann. N. Y. Acad. Sci. 386, 314.

(52) Lazarow,P.B., Robbi,M., Fujiki,Y. & Wong,L. (1982) Ann. N. Y. Acad. Sci. 386, 285.

(53) Neupert,W. & Shatz,G. (1981) Trends Biochem. Sci. 6, 1.

(54) Yamada,T., Tanaka,A., Horikawa,S., Numa,S. & Fukui,S. (1982) Eur. J. Biochem. 129, 251.

(55) Furuta,S., Hashimoto,T., Miura,S., Mori,M. & Tachibana,M. (1982) Biochem. Biophys. Res. Commun. 105, 639.

(56) Hock,B. & Gietl,C. (1982) Ann. N. Y. Acad. Sci. 386, 350.

(57) Ueda,M., Tanaka,A., Horikawa,S., Numa,S. & Fukui,S. (1984) Eur. J. Biochem. 138, 451.

(58) Shimizu,S., Yasui,K., Tani,Y. & Yamada,H. (1979) Biochem. Biophys. Res. Commun. 91, 108.

(59) Coudron,P,E., Frerman,F.E. & Schowalter,P.B. (1983) Arch. Biochem. Biophys. 226, 324.

(60) Jiang,Z. & Thorpe,C. (1983) Biochemistry 22, 3752.

(61) Yamada,T., Tanaka,A. & Fukui,S. (1982) Eur. J. Biochem. 125, 517.

(62) Ueda,M., Tanaka,A. & Fukui,S. (1984) Eur. J. Biochem. 138, 445.

SYNOPSIS

Part I Peroxisomal fatty acid β-oxidation system in n-alkane-utilizing yeast, Candida tropicalis

In the yeast, Candida tropicalis, peroxisomes profusely appear in the cells grown on alkanes. These organelles contain the several enzymes related to fatty acid β-oxidation system and glyoxylate cycle, playing an indispensable role on alkane assimilation.

Chapter 1 deals with the subcellular localization of four enzymes related to the fatty acid β-oxidation system in the yeast. Although fatty acid β-oxidation system was found to be localized specifically in peroxisomes of C. tropicalis cells growing on n-alkanes based on the stoichiometric study of palmitoyl-CoA-dependent activities of NAD reduction, acetyl-CoA formation and oxygen consumption in the presence or absence of sodium azide, an inhibitor of catalase, the induction and the localization of each enzyme which constitutes the system has not been investigated. The author has proved that four enzymes of the fatty acid β-oxidation system ----- acyl-CoA oxidase,

enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase ----- were induced by alkanes and localized only in peroxisomes but not in mitochondria of alkane-grown C. tropicalis, and that acyl-CoA dehydrogenase, the enzyme catalyzing the initial step of the mitochondrial β-oxidation in mammalian cells, could not be detected in any subcellular fractions from the yeast under the conditions employed. These results indicated that the peroxisomal β-oxidation system exclusively participates in the degradation of fatty acids derived from alkanes and in the formation of acetyl-CoA.

Chapter 2 deals with the relationship between enoyl-CoA hydratase and a peroxisomal bifunctional enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, from an n-alkane-utilizing yeast, C. tropicalis. The yeast β-oxidation system was found to be composed of acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. In mammalian cells and plant cells, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were completely separable enzymes in the mitochondrial β-oxidation system, while both enzyme activities in peroxisomes were involved in a bifunctional

protein of a single peptide. It is interesting whether peroxisomal enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase in the yeast are composed of a single peptide having these enzyme activities or are present as separate peptides. From an n-alkane-assimilating yeast, C. tropicalis, the author purified the enzyme, composed of 36 kDa subunits with only enoyl-CoA hydratase activity, and also purified, in the presence of protease inhibitors, a bifunctional enzyme, composed of 105 kDa monomeric form with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. From the comparison of peptide maps between the purified enoyl-CoA hydratase and the 36 kDa fragment obtained by limited proteolysis from the bifunctional enzyme, enoyl-CoA hydratase was shown to be derived from the bifunctional enzyme, the results indicating that the enoyl-CoA hydratase domain was separable from the bifunctional enzyme by the action of a certain protease.

Part II Glyoxylate cycle enzymes, isocitrate lyase and malate synthase, of n-alkane-utilizing Candida tropicalis

Isocitrate lyase and malate synthase are the key enzymes in the glyoxylate cycle. In alkane-grown cells of C. tropicalis, isocitrate lyase and malate synthase are induced and localized in peroxisomes.

Although, in plant cells, both enzymes which were localized in glyoxysomes in germinating seeds are purified and characterized, and in vitro incorporation of these enzymes into glyoxysomes has been examined, little information is available concerning these enzymes in yeasts.

The auther has investigated the properties of isocitrate lyase purified not only from glucose-grown C. tropicalis cells but also from peroxisomes of alkane-grown cells. The results are summarized in chapter 1.

In C. tropicalis, the activity level of isocitrate lyase in alkane-grown cells was considerably higher than in glucose-grown cells. Most part of the enzyme was localized in peroxisomes when the yeast was grown on alkanes. A large part of the constitutive enzyme in glucose-grown cells was also localized in pre-existing peroxisomes. This indicated that isocitrate lyase could be of use as a target enzyme for investigating the biogenesis and development of yeast peroxisomes through the mechanism of enzyme localization in

the organelles. The enzymes purified from glucose-grown cells and the peroxisome-containing fraction from alkane-grown cells had a molecular mass of 130 kDa, composed of two identical subunits of a molecular mass of 65 kDa, showed similar peptide maps upon partial digestion with proteolytic enzymes, and were indistinguishable immunochemically. These results suggested that both enzymes are products of the same gene.

Chapter 2 deals with purification of peroxisomal malate synthase and some properties of the purified enzyme.

Malate synthase was purified from peroxisomes of alkane-grown C. tropicalis cells. The enzyme, which was localized in peroxisomal matrix, had a molecular mass of 250 kDa, composed of four identical subunits of a molecular mass of 61 kDa. Several properties of this enzyme were also investigated.

In chapter 3, comparison and subcelluar distribution of isocitrate lyase and malate synthase in C. tropicalis cells grown on different carbon sources are described. C. tropicalis cells grown on n-alkanes as the sole source of carbon and energy have profuse numbers of peroxisomes. This yeast can utilize glucose, acetate and propionate as

carbon sources. The cells grown on glucose or acetate have a few peroxisomes, that is, pre-existing peroxisomes. In contrast to alkane-grown cells containing a large number of small peroxisomes, the cells grown on propionate have been observed to have a small number of large peroxisomes, which may be regarded as immature peroxisomes. The activities of isocitrate lyase and malate synthase were fairly high in n-alkane-, acetate-, and propionate-grown cells compared with those in glucose-grown cells. Both enzymes were localized predominantly in mature peroxisomes of alkane-grown cells and pre-existing peroxisomes of glucose-grown cells, while the cytosolic enzymes were predominant in propionate-grown cells. In acetate-grown cells, these enzymes were localized in pre-existing peroxisomes as well as in cytosol. Isocitrate lyase and malate synthase were purified from a peroxisome-containing particulate fraction of alkane-grown cells and from whole cells grown on glucose, acetate, and propionate. The respective enzymes showed no significant differences in catalytic, proteinaceous, and immunochemical properties. The results indicated that the different forms of the active and inactive enzymes were not present in the yeast cells.

Part III Purification of a novel propionate-activating (propionylhydroxamate-forming) enzyme from propionate-grown cells of *Candida tropicalis*

Candida tropicalis grown on *n*-alkanes or higher fatty acids has conspicuous numbers of peroxisomes, which play an indispensable role in the fatty acid metabolism. As the final products of peroxisomal β-oxidation of fatty acids with even and odd carbon chains are acetyl-CoA and propionyl-CoA, it will be of interest to investigate the roles of peroxisomes in the metabolism of C_2- and C_3-compounds together with the development of the organelles. In the course of these studies, the author has found out the presence of a novel propionate-activating enzyme, which was different from acetyl-CoA synthetase and induced in cytoplasm of propionate-grown cells. The enzyme consisted of a single peptide of a molecular mass of 87 kDa and exhibited a pH-optimum of 5.5. In addition to propionate, acetate, butyrate and valerate served as substrates. The formation of hydroxamates was independent of ATP and CoA. In contrast, acetyl-CoA synthetase constitutively present in

the cytoplasm of acetate- and propionate-grown yeast cells had a subunit molecular mass of 72 kDa and a pH-optimum of 7.5. Activation of acetate and propionate by this enzyme required ATP and CoA. This observation suggests that the propionate-activating enzyme has a reaction mechanism different from that of the synthetase.

Part I. Peroxisomal fatty acid β-oxidation system in n-alkane-utilizing Candida yeast

Chapter 1. Peroxisomal localization of enzymes related to fatty acid β-oxidation in n-alkane-grown yeast, Candida tropicalis

INTRODUCTION

Candida tropicalis assimilating n-alkanes is one of the valuable microorganisms to study fatty acid metabolism in eukaryotic cells (1). Peroxisomes (microbodies) profusely appeared in the n-alkane-grown yeast cells (2,3) have been revealed to participate in the metabolism of alkanes, based on the subcellular localization of various enzymes in the cells (4-6). Professor Fukui's group have first demonstrated that the fatty acid β-oxidation system was localized in the yeast peroxisomes of alkane-grown C. tropicalis cells, from the stoichiometric study on the reduction of NAD, the formation of acetyl-CoA and hydrogen peroxide and the consumption of oxygen depending on fatty

acyl-CoA in the presence or absence of sodium azide, an inhibitor of catalase (7). Under the conditions employed, the β-oxidation system was not found in mitochondria. Although acyl-CoA oxidase (EC 1.3.99.3) was purified from whole cells of C. tropicalis and characterized (8), no information is available about the induction and the localization of respective enzymes of the fatty acid β-oxidation system in the yeast.

β-oxidation system has been demonstrated in microbodies of castor bean (9), Tetrahymena (10) and Euglena (11). Many studies on peroxisomal and mitochondrial β-oxidation systems have been reported with mammalian cells treated with peroxisome-proliferators such as clofibrate (12-14). The peroxisomal system plays a distinct role in degrading long chain fatty acids to middle chain fatty acids, and the mitochondrial system in degrading middle chain fatty acids, transported from peroxisomes, to acetyl-CoA. In addition, three enzymes constituting the peroxisomal fatty acid β-oxidation system ----- enoyl-CoA hydratase (EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) ----- have been demonstrated to be immunochemically distinguishable from those in the

mitochondrial system. There also exist differences between the peroxisomal system and the mitochondrial system, that is, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase in peroxisomes constitute a bifunctional protein, while these enzymes in mitochondria are different proteins, and the initial step of the β-oxidation in peroxisomes is mediated by acyl-CoA oxidase but that in mitochondria by acyl-CoA dehydrogenase.

Chapter 1 deals with the induction and subcellular localization of four enzymes constituting the fatty acid β-oxidation system in alkane-grown yeast, C. tropicalis. The results obtained indicated that the respective enzymes were peroxisomal.

MATERIALS AND METHODS

1. Cultivation of yeast.

The yeast used in this study was Candida tropicalis (Castellani) Berkhout strain pK 233 (ATCC 20336), which was maintained on an agar slant of a glucose medium. The organism was cultured as follows. Cells, precultured aerobically for 22 h in 1.65 % glucose medium, were

inoculated in a flask containing an alkane medium, and incubated for 17 h at 30 °C with shaking (220 rev./min). The alkane medium was composed of 5.0 g of $NH_4H_2PO_4$, 2.5 g of KH_2PO_4, 1.0 g of $MgSO_4.7H_2O$, 0.02 g of $FeCl_3.6H_2O$, 1.0 ml of corn steep liquor, 0.5 ml of of Tween 80 and 10 ml of n-alkane mixture per liter of tap water (pH 5.2). n-Alkane mixture used as carbon and energy source was "mixture 11", composed of C_{10} 16.4, C_{11} 50.4, C_{12} 32.5 and C_{13} 0.7 % by weight. The glucose medium contained 1.65 % (w/v) glucose in place of n-alkane mixture and Tween 80 (15).

2. Preparation of enzyme sources

Cells harvested by centrifugation at 1,000 x g for 5 min were washed twice with deionized water and once with 50 mM potassium phosphate buffer (pH 7.2). Washed cells were suspended in the same buffer and homogenized with a Braun homogenizer (for 150 s per 1.5 g dry cells). The homogenized cell suspension was centrifuged at 10,000 x g for 15 min and then ultracentrifuged at 127,000 x g for 1 h. The supernatant obtained was used as the cell-free extracts.

3. Subcellular fractionation

Yeast cells (1.5 g dry cells) harvested at the exponential growth phase were suspended in 200 ml of 40 mM potassium phosphate buffer (pH 7.2) containing 0.65 M sorbitol, and treated with 40 mg of a bacterial lytic enzyme, "Zymolyase 20T" for 70 - 80 min at 30 °C until the degree of lysis reached about 75 %. Hereafter all the procedures were carried out at 0 - 4°C. The protoplasts so formed were collected by centrifugation at 3,000 x g for 10 min, suspended in 20 ml of 2.5 mM potassium phosphate buffer (pH 7.2) containing 0.65 M sorbitol and 0.5 mM EDTA and homogenized for 10 min (20 times up and down) in a teflon homogenizer under cooling. The homogenate was centrifuged at 3,000 x g for 10 min to remove heavy particles (P_1). The supernatant (S_1) was subjected to centrifugation again at 20,000 x g for 15 min to obtain supernatant fraction (S_2) and particulate fraction (P_2) containing peroxisomes and mitochondria. P_2 fraction was suspended in 8.0 ml of 2.5 mM potassium phosphate buffer (pH 7.2) containing 20.0 % sucrose and 0.5 mM EDTA and an aliquot (2.5 ml) was layered over the discontinuous sucrose density gradient composed of the same buffer containing

30.0, 40.0, 41.3, 42.5 and 50.0 % sucrose (each 2.5 ml). Intact peroxisomes and mitochondria were isolated by centrifugation at 49,600 x g for 2 h. The fraction containing catalase was the peroxisomal fraction and that containing cytochrome oxidase was the mitochondrial fraction.

4. Enzyme assay

Catalase [EC 1.11.1.6] was assayed spectrophotometrically by the same method described by Roggenkamp et al. (16,17). Cytochrome oxidase [EC 1.9.3.1] was measured by the method of Polakis et al. (18). Protein was determined by Lowry's method (19). Overall activity of the β-oxidation system was measured by following the change in absorbance of NADH at 340 nm according to the method of Professor Fukui's group (7) with a slight modification. The reaction mixture contained 27.5 mM potassium phosphate buffer (pH 7.2), 5 μM palmitoyl-CoA or decanoyl-CoA, 5 μM L-carnitine, 0.3 mM CoA, 1.0 mM NAD, 1.0 mM $MgCl_2$, 3.3 mM NaN_3, 10 mM dithiothreitol and enzyme preparation in a final volume of 2.0 ml.

Acyl-CoA oxidase activity was determined by following the increase in absorbance of a quinoneimine dye depending

on palmitoyl-CoA or decanoyl-CoA at 500 nm (20). The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.4), 0.83 mM 4-aminoantipyrine, 10.6 mM phenol, 10 μM FAD, 0.1 mM palmitoyl-CoA or decanoyl-CoA, 45 U peroxidase (from horse radish) and enzyme preparation in a final volume of 1.5 ml.

Acyl-CoA dehydrogenase activity was assayed by following the decrease in absorbance of dichlorophenol-indophenol depending on palmitoyl-CoA or decanoyl-CoA at 600 nm (21). The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.5), 30 μM L-carnitine, 1.0 mM N-ethylmaleimide, 3.0 μM palmitoyl-CoA or decanoyl-CoA, 35 μM dichlorophenolindophenol, 1.6 mM phenazine methosulfate and enzyme preparation in a final volume of 1.5 ml.

Enoyl-CoA hydratase activity was measured by following the decrease in absorbance of crotonyl-CoA at 263 nm (22). The reaction mixture contained 83.4 mM potassium phosphate buffer (pH 8.0), 25 μM crotonyl-CoA and enzyme preparation in a final volume of 1.5 ml. Enoyl-CoAs with long chain of carbon are not available commercially. Therefore, an alternative novel method coupled with acyl-CoA oxidase was devised (Fig. 1). Palmitoyl-CoA or decanoyl-CoA was first

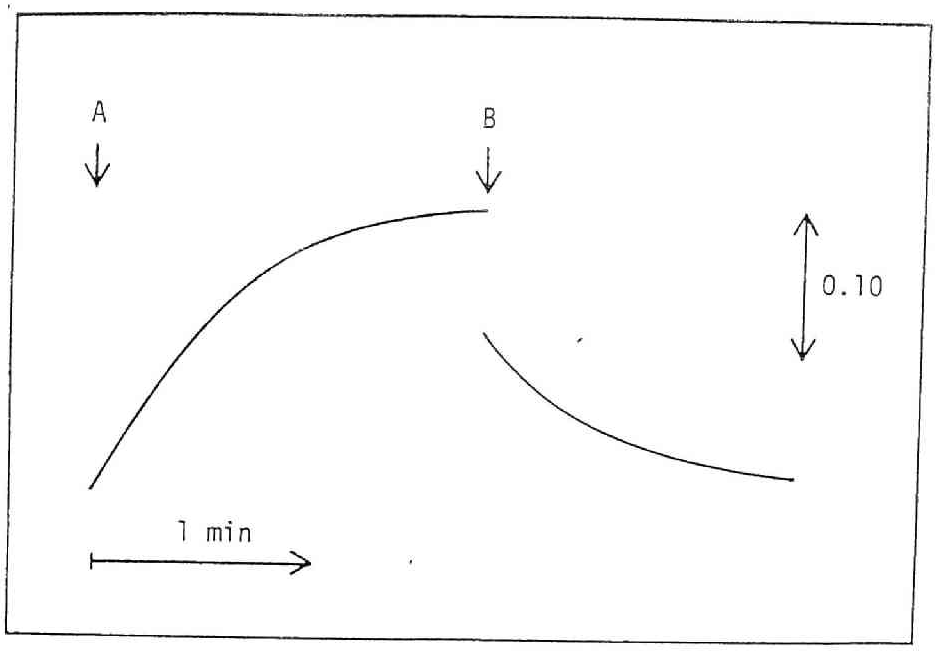


Fig. 1. Novel assay system for enoyl-CoA hydratase

The initial reaction mixture contained 83.2 mM potassium phosphate buffer (pH 8.0), 25 μM decanoyl-CoA and 1.68 ng of acyl-CoA oxidase in a volume of 1.4 ml. The reaction was started by the addition of acyl-CoA oxidase (at A). After the absorbance of enoyl-CoA at 263 nm reached a plateau, the mixture was boiled for 10 min and cooled to 30 °C, and enzyme preparation from Candida tropicalis (0.1 ml) was added to the mixture (at B). Then, the decrease in the absorbance at 263 nm was followed. The extinction coefficient for crotonyl-CoA (19) was applied for the estimation of the enzyme activity. In this experiment P_2 fraction was used as enzyme preparation. Palmitoyl-CoA used as substrate gave a similar result.

dehydrogenated to the corresponding enoyl-CoA with acyl-CoA oxidase (checked by the increase in absorbance at 263 nm). After the reaction mixture was boiled and cooled, enzyme preparation from C. tropicalis was added to the mixture and the decrease in absorbance of enoyl-CoA was followed at 263 nm. Formation of enoyl-CoA during the second reaction was not taken into account because the activity of acyl-CoA oxidase in the enzyme preparation from C. tropicalis was lower than that of enoyl-CoA hydratase. As this method gave a similar result to that with crotonyl-CoA, it seemed to be much valid to measure the activity of enoyl-CoA hydratase with enoyl-CoAs of different carbon chains. Unless mentioned otherwise, this novel method was applied for the assay of enoyl-CoA hydratase in this study.

3-Hydroxyacyl-CoA dehydrogenase activity was determined by following the decrease in absorbance of NADH depending on acetoacetyl-CoA at 340 nm (23). The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.4), 25 μM acetoacetyl-CoA, 0.1 mM NADH and enzyme preparation in a final volume of 1.5 ml.

3-Ketoacyl-CoA thiolase activity was assayed by following the decrease in absorbance of acetoacetyl-CoA

depending on CoA at 303 nm (24,25). The reaction mixture contained 100 mM Tris/HCl buffer (pH 8.3), 25 mM $MgCl_2$, 50 mM KCl, 40 μM acetoacetyl-CoA, 50 μM CoA and enzyme preparation in a final volume of 1.5 ml.

All the assays were carried out at 30 °C.

5. Chemicals

NAD and CoA were gifted from Kohjin Co. (Tokyo, Japan). Other CoA derivatives were obtained from Sigma (St. Louis, USA) and acyl-CoA oxidase was donated from Amano Seiyaku Co. (Nagoya, Japan). Zymolyase 20T was obtained from Seikagaku Kogyo Co. (Tokyo, Japan) and other chemicals were purchased from commercial sources.

RESULTS

1. Induction of enzymes related to fatty acid β-oxidation system

The levels of the respective enzyme activities constituting fatty acid β-oxidation system in Candida tropicalis cells grown to the exponential phase on n-alkanes and glucose are presented in Table 1. High levels

Table 1. Levels of enzymes related to fatty acid β-oxidation system in glucose-grown and n-alkane-grown Candida tropicalis pK 233

Enzyme activities were measured with cell-free extracts prepared from cells harvested at the exponential growth phase.

Enzyme	Enzyme activity ($nmol.min^{-1}.mg\ protein^{-1}$)		(B)/(A)
	Glucose-grown cells (A)	Alkane-grown cells (B)	
Acyl-CoA dehydrogenase	ND[a]	ND	—
Acyl-CoA oxidase	6.2	2500	403
Enoyl-CoA hydratase[b]	55	5700	104
3-Hydroxyacyl-CoA dehydrogenase	ND	240	—
3-Ketoacyl-CoA thiolase	67	190	2.8

[a] Not detectable

[b] Crotonyl-CoA was used as substrate.

of acyl-CoA oxidase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities were observed in alkane-grown cells compared with those in glucose-grown cells. The level of 3-ketoacyl-CoA thiolase activity was about three times higher in the cells grown on alkanes than that in glucose-grown cells. Acyl-CoA dehydrogenase, which is known as the enzyme participating in the initial step of the mitochondrial β-oxidation in mammalian cells, was not detected in both cells.

2. Subcellular localization of enzymes related to β-oxidation system

Alkane-grown cells of C. tropicalis exhibited the activities of the enzymes related to the fatty acid β-oxidation system, as described above, as well as the overall activity of the system. These activities were mainly found in the particulate fraction P_2 (20,000 x g pellets) containing catalase, a marker enzyme of peroxisomes, and cytochrome oxidase, that of mitochondria (Fig. 2). The activities observed in S_2 fraction (20,000 x g supernatant) might be derived from the organelles disintegrated during preparation of the subcellular fractions. Acyl-CoA

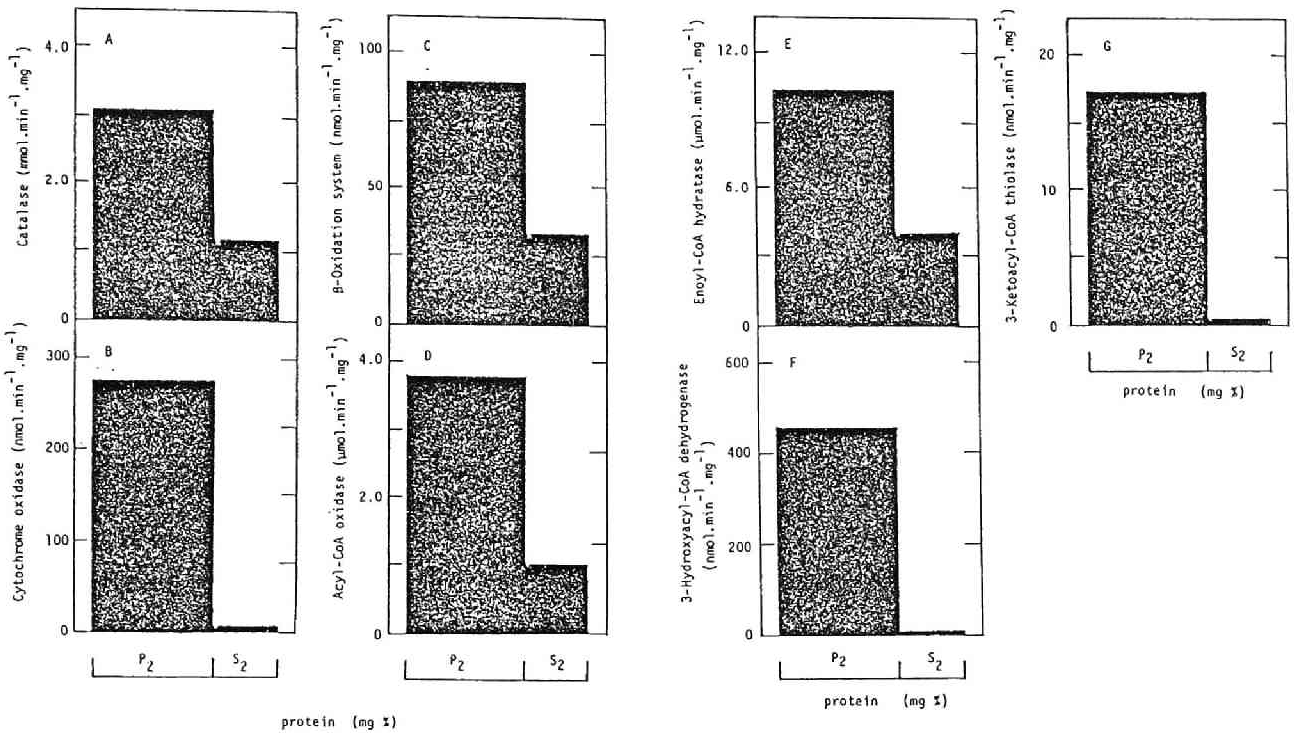


Fig. 2. Subcellular localization of enzymes in n-alkane-grown Candida tropicalis.

The experimental procedures were described in "MATERIALS AND METHODS". P_2, 20,000 x g pellets (peroxisomes and mitochondria); S_2, 20,000 x g supernatant (microsomes and cytoplasm). A, catalase; B, cytochrome oxidase; C, β-oxidation system assayed with decanoyl-CoA; D, acyl-CoA oxidase assayed with decanoyl-CoA; E, enoyl-CoA hydratase assayed as in Fig. 1; F, 3-hydroxyacyl-CoA dehydrogenase; G, 3-ketoacyl-CoA thiolase.

dehydrogenase was detected neither in P_2 fraction nor S_2 fraction under the conditions employed. When decanoyl-CoA or palmitoyl-CoA was used as substrate to measure the activities of overall β-oxidation system, acyl-CoA oxidase and acyl-CoA dehydrogenase, no difference was observed between the results with these substrates.

To obtain more detailed information about the subcellular localization of these enzymes, it is necessary to isolate intact peroxisomes and mitochondia by sucrose density gradient centrifugation of P_2. The buffer concentration in the protoplast homogenate and in the sucrose gradient was found to affect the separation of these organelles. With 2.5 mM potassium phosphate buffer, instead of 50 mM potassium phosphate buffer employed before, good separation of peroxisomes and mitochondria and high reproducibility of the results were obtained. This procedure enabled us to demonstrate that acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, the respective enzymes constituting the fatty acid β-oxidation system, were localized only in peroxisomes of alkane-grown cells of C. tropicalis (Fig. 3). Acyl-CoA dehydrogenase was not detected again in these organelles.

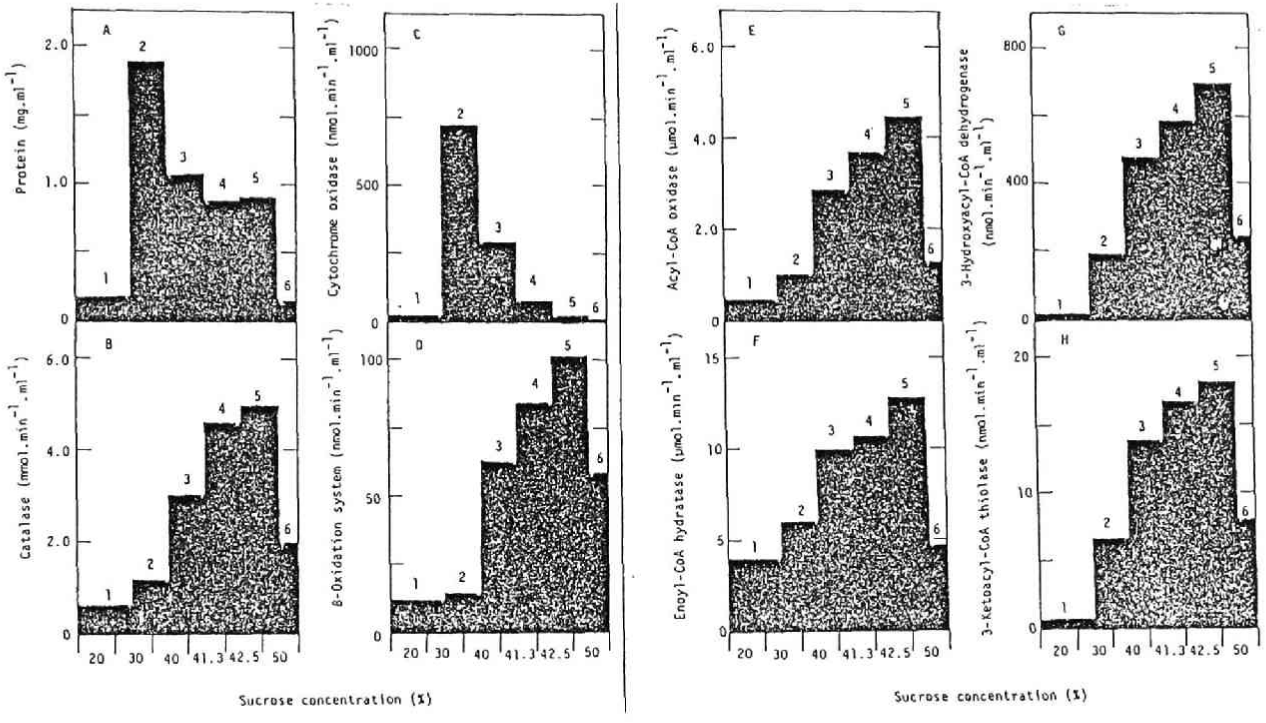


Fig. 3. Particulate localization of enzymes in P_2.

The experimental procedures were described in "MATERIALS AND METHODS". The volume of each fraction was as follows: 1, 3.75 ml; 2 - 5, 2.5 ml each; 6, 1.25 ml. A, protein; B, catalase; C, cytochrome oxidase; D, β-oxidation system; E, acyl-CoA oxidase; F, enoyl-CoA hydratase; G, 3-hydroxyacyl-CoA dehydrogenase; H, 3-ketoacyl-CoA thiolase. Enzyme activities were measured as in Fig. 2.

DISCUSSION

Peroxisomes and mitochondria have recently become very significant organelles in studying the biology of eukaryotic cells (26). Peroxisomes are one of the organelles whose development can be controlled under various conditions, and can give a model to investigate the transportation of precursor proteins encoded by nuclear genes. In mammalian cells, in which peroxisomes develop after the administration of various hypolipidemic drugs including clofibrate etc., catalase and fatty acid β-oxidation enzymes have been found in the organelles and the role of the peroxisomal β-oxidation system in the cells has been discussed in detail.

With yeasts, presence of the fatty acid β-oxidation system was first demonstrated by Professor Fukui's group in peroxisomes developed in the cells grown on n-alkanes or higher fatty acids as the sole source of carbon and energy (7,27). The yeast system was proved to be similar to that of mammalian peroxisomes from the stoichiometric study among NAD reduction, oxygen consumption, acetyl-CoA formation and hydrogen peroxide formation. That is, the initial step of

the system in Candida tropicalis is catalyzed by acyl-CoA oxidase yielding hydroden peroxide, which is degraded by peroxisomal catalase (7). However, the individual enzymes constituting the system, except for acyl-CoA oxidase (8), have not been detected in the yeast cells, and their localization in peroxisomes has not been clarified yet.

Peroxisomes and mitochondria were separated successfully by using potassium phosphate buffer of a low concentration for the preparation of protoplast homogenate and for the sucrose density gradient centrifugation. The purity of the organelles seemed to be enough to conclude the subcellular localization of the β-oxidation enzymes, judged from the distribution of the respective marker enzymes (catalase and cytochrome oxidase).

Thus, it could be first demonstrated that four enzymes of the fatty acid β-oxidation system ----- acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase ----- were induced by alkanes and localized only in peroxisomes but not in mitochondria of alkane-grown C. tropicalis, and that acyl-CoA dehydrogenase, the enzyme catalyzing the initial step of the

mitochondrial β-oxidation system in mammalian cells, could not be detected in any subcellular fractions from the yeast under the conditions employed. These results indicated that fatty acids derived from alkanes are degraded to acetyl-CoA exclusively by the peroxisomal β-oxidation system. The metabolism of fatty acids in yeast is different from that in mammalian cells, in which long chain fatty acyl-CoAs are metabolized to middle chain acyl-CoAs by the peroxisomal β-oxidation system and the latters are degraded to acetyl-CoA by the mitochondrial system. Acetyl-CoA so formed in yeast peroxisomes might be transported to mitochondria by the function of peroxisomal and mitochondrial carnitine acetyltransferases (28) for the operation of TCA cycle in mitochondria and glyoxylate cycle between peroxisomes and mitochondria (Fig. 4).

The enzymes related to the fatty acid β-oxidation system might be precious targets to study the biogenesis and development of yeast peroxisomes as well as the localization of proteins and the topography of the individual enzymes localized in peroxisomes (29,30), because these enzymes are inducibly synthesized during alkane assimilation by yeast and efficiently located into peroxisomes.

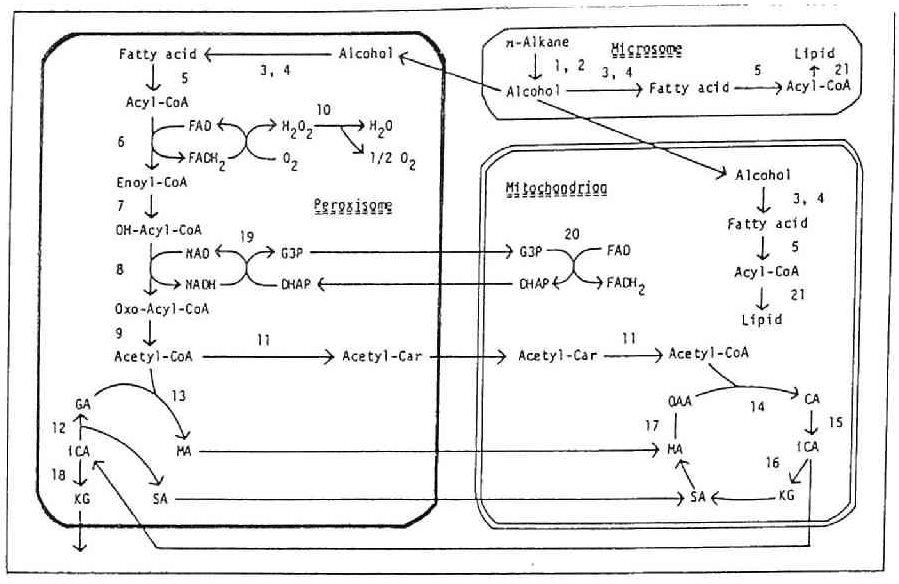


Fig. 4. Presumptive roles of peroxisomes, mitochondria and microsomes in n-alkane metabolism by Candida tropicalis.

Enzyme: 1, cytochrome P-450; 2, NADPH-cytochrome P-450 (cytochrome c) reductase; 3, long-chain alcohol dehydrogenase; 4, long-chain aldehyde dehydrogenase; 5, acyl-CoA synthetase; 6, acyl-CoA oxidase; 7, enoyl-CoA hydratase; 8, 3-hydroxyacyl-CoA dehydrogenase; 9, 3-ketoacyl-CoA thiolase; 10, catalase; 11, carnitine acetyltransferase; 12, isocitrate lyase; 13, malate synthase; 14, citrate synthase; 15, aconitase; 16, NAD-linked isocitrate dehydrogenase; 17, malate dehydrogenase; 18, NADP-linked isocitrate dehydrogenase; 19, NAD-linked glycerol-3-phosphate dehydrogenase; 20, FAD-linked glycerol-3-phosphate dehydrogenase; 21, glycerol-3-phosphate acyltransferase. Abbreviations used: Acetyl-Car, acetylcarnitine; CA, citrate; DHAP, dihydroxyacetone phosphate; GA, glyoxylate; G3P, glycerol-3-phosphate; iCA, isocitrate; KG, 2-ketoglutarate; MA, malate; OAA, oxalacetate; OH-acyl-CoA, 3-hydroxyacyl-CoA; Oxo-acyl-CoA, 3-ketoacyl-CoA; SA, succinate.

SUMMARY

In Candida tropicalis cells grown on n-alkanes (C_{10}- C_{13}), the levels of the enzyme activities related to the fatty acid β-oxidation -----acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase ----- were found to be higher than those in cells grown on glucose, indicating that these enzymes were induced by alkanes. These enzymes were first confirmed to be localized only in peroxisomes, while none of these enzymes nor acyl-CoA dehydrogenase, which is known to participate in the initial step of the mitochondrial β-oxidation in mammalian cells, were detected in yeast mitochondria under the conditions employed.

The significance of the peroxisomal β-oxidation system in the metabolism of alkanes by yeast was also discussed.

REFERENCES

(1) Ueda,M., Tanaka,A. & Fukui,S. (1982) Eur. J. Biochem. 124, 205.

(2) Osumi,M., Miwa,N., Teranishi,Y., Tanaka,A. & Fukui,S. Arch. Microbiol. (1974) 99, 181.

(3) Osumi,M., Fukuzumi,F., Teranishi,Y., Tanaka,A. & Fukui,S. Arch. Microbiol. (1975) 103, 1.

(4) Fukui,S. & Tanaka,A. (1979) J. Appl. Biochem. 1, 171.

(5) Fukui,S. & Tanaka,A. (1979) Trends Biochem. Sci. 4, 246.

(6) Tanaka,A., Osumi,M. & Fukui,S. (1982) Ann. N. Y. Acad. Sci. 386, 183.

(7) Kawamoto,S., Nozaki,C., Tanaka,A. & Fukui,S. (1978) Eur. J. Biochem. 83, 609.

(8) Shimizu,S., Yasui,K., Tani,Y. & Yamada,H. (1979) Biochem. Biophys. Res. Commun. 91, 108.

(9) Cooper,T.G. & Beevers,H. (1969) J. Biol. Chem. 244, 3514.

(10) Blum,J.J. (1973) J. Protozool. 20, 688.

(11) Graves,Jr.,L.B. & Becker,W.M. (1974) J. Protozool. 21, 771.

(12) Lazarow,P.B. & de Duve,C. (1976) Proc. Natl. Acad. Sci. USA 73, 2043.

(13) Masters,C. & Crane,D. (1984) Trends Biochem. Sci. 7, 314.

(14) Osumi,T. & Hashimoto,T. (1984) Trends Biochem. Sci. 7, 317.

(15) Tanaka,A., Yamamura,M., Kawamoto,S. & Fukui,S (1977) Appl. Environ. Microbiol. 34, 342.

(16) Fukui,S., Kawamoto,S., Yasuhara,S., Tanaka,A., Osumi,M. & Imaizumi,F. (1975) Eur. J. Biochem. 59, 561.

(17) Roggenkamp,R., Sahm,H. & Wadner,F. (1974) FEBS Lett. 41, 283.

(18) Polakis,E.S., Barthey,W. & Melk,G.A. (1964) Biochem. J. 90, 369.

(19) Lowry,O.H., Rosebrough,N.J., Farr,A.L. & Randall,R.J. (1951) J. Biol. Chem. 193, 265.

(20) Allain,C.C., Poon,L.S., Chan,C.S.G., Richmond,W. & Fu,P.C. (1974) Clin. Chem. 20, 470.

(21) Furuta,S., Miyazawa,S. & Hashimoto,T. (1981) J. Biochem. 90, 1739.

(22) Shultz,H. (1974) J. Biol. Chem. 249, 2704.

(23) Osumi,T. & Hashimoto,T. (1979) Biochim. Biophys. Acta 574, 258.

(24) Staak,H., Binstock,J.F. & Shultz,H. (1978) J. Biol. Chem. 253, 1827.

(25) Miyazawa,S., Osumi,T. & Hashimoto,T. (1980) Eur. J. Biochem. 103, 589.

(26) de Duve,C. (1983) Scientic American 248, 52.

(27) Mishina,M., Kamiryo,T., Tashiro,S., Hagihara,T., Tanaka,A., Fukui,S., Osumi,M. & Numa,S. (1978) Eur. J. Biochem. 89, 321.

(28) Ueda,M., Tanaka,A. & Fukui,S. (1984) Eur. J. Biochem. 138, 445.

(29) Blobel,G. (1980) Proc. Natl. Acad. Sci. USA 77, 1496.

(30) Hayashi,H., Hino,S. & Yamasaki,F. (1981) Eur. J. Biochem. 120, 47.

Chapter 2. Relationship between enoyl-CoA hydratase and a peroxisomal bifunctional enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, from an n-alkane-utilizing yeast, Candida tropicalis

INTRODUCTION

Mammalian cells have two types of β-oxidation systems: One is the instinctive mitochondrial, another is the inducible peroxisomal systems (1). Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were completely separable enzymes in the mitochondrial β-oxidation system, while both enzyme activities in peroxisomes were involved in a bifunctional protein of a single peptide (2,3) as the case of plant cells (4). The peroxisomal bifunctional enzyme has become significant as a target protein on the investigation of the differences in reaction mechanism and evolution, and differentiation of the function into peroxisomal and mitochondrial β-oxidation systems.

As described in Chapter 1, peroxisomes from an n-alkane-grown yeast, Candida tropicalis, contain the

complete fatty acid β-oxidation system but mitochondria do not. The yeast β-oxidation system was found to be composed of acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. It is interesting whether peroxisomal enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase in the yeast are composed of a single peptide having these enzyme activities or are present as separate peptides. Moreno de la Garza et al. (5) purified a multifunctional enzyme exhibiting the activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase from oleate-grown C. tropicalis. In the meantime, enzymes having only enoyl-CoA hydratase activity and having both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities were obtained from alkane-grown C. tropicalis under the different conditions of purification.

Chapter 2 describes the comparison of the properties and the relationship between enoyl-CoA hydratase and the bifunctional enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase.

MATERIALS AND METHODS

1. Cultivation of yeast

Candida tropicalis pK 233 (ATCC 20336) were cultivated on alkanes as in Chapter 1 (6).

2. Preparation of enzyme sources

(A) Cell-free extract to purify enoyl-CoA hydratase was prepared from the cells harvested at the exponential growth phase by disintegrating the cells with a Braun homogenizer as in Chapter 1.

(B) Yeast protoplasts were prepared as in Chapter 1 in the presence of 0.1 M 2-mercaptoethanol. Protoplasts were homogenized with a teflon homogenizer and fractionated by differential centrifugations. The P_2 fraction (20,000 x g pellets) containing peroxisomes and mitochondria was treated under hypotonic conditions in the presence of protease inhibitors and a detergent, and the supernatant (P_2-S fraction) obtained by centrifugation (127,000 x g, 1 h) was used as the source of a bifunctional enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase.

3. Electrophoresis and limited proteolysis

Polyacrylamide slab-gel electrophoresis in the presence of sodium dodecylsulfate was performed as described by King and Laemmli (7) except that both the stacking and the separating gels contained 0.25 mM EDTA. Limited proteolysis with proteases was carried out as follows: The indicated bands dissociated on 10 % polyacylamide slab-gel in the presence of sodium dodecylsulfate were excised and electrophoresed with Staphylococus aureus V8 protease or papain as described by Cleveland et al. (8), except that 15 % acrylamide gels were used as the digesting gels. Peptide fragments were detected by the silver staining method. (Technical Manual I, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan)

4. Determination of molecular masses of native forms

Gel filtration to measure the molecular masses of the native forms of the enzymes was carried out under the following conditions. The enzyme dissolved in 10 mM potassium phosphate buffer (pH 7.2) containing 0.3 M KCl was applied to a Sepharose 6B column (1.5 x 65 cm) equilibrated with the above buffer, and eluted with the elution buffer of

10 mM potassium phosphate buffer (pH 7.2) containing 0.3 M potassium chloride. Thyroglobulin, catalase, aldolase, bovine serum albumin, ovalbumin and cytochrome c were used as markers of molecular weight.

5. Enzyme assay

Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity was measured as described in Chapter 1.

6. Chemicals

Hydroxyapatite (Hypatite) HCA 100-S was kindly donated by Mitsui Toatsu Chemicals (Tokyo, Japan) and Amphitol 20B(S) from Kao Atlas (Tokyo, Japan). DEAE-Sephacel, Sephacryl S-300, DEAE-Sepharose CL-6B, and molecular mass kit were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), Cellulofine GCL-2000-m from Seikagaku Kogyo (Tokyo, Japan), phenylmethylsulfonyl fluoride, CoA derivatives and electrophoresis calibration kit for molecular mass from Sigma (St. Louis, MO, USA), and silver staining reagents from Daiichi Pure Chemicals (Tokyo, Japan). Other chemicals were also purchased from commercial sources.

RESULTS

1. Purification of enoyl-CoA hydratase

The enzyme was purified from the cell-free extract by the successive chromatographies on a hydroxyapatite column (3.7 x 18 cm) with a linear concentration gradient of potassium phosphate buffer prepared from 50 mM and 500 mM potassium phosphate buffer (pH 7.2), on a Cellulofine GCL-2000-m column (1.9 x 40 cm) with 50 mM potassium phosphate buffer (pH 7.2), and on a DEAE-Sephacel column (2.1 x 25 cm) with a linear concentration gradient of KCl prepared from 50 mM potassium phosphate buffer (pH 7.2) containing 0.27 M KCl and the same buffer containing 0.40 M KCl. The results are summarized in Table 1.

The ratio of 3-hydroxyacyl-CoA dehydrogenase activity to enoyl-CoA hydratese activity decreased to nil along with the progress of the purification. The molecular mass of the subunit of this enzyme was determined to be 36 kDa on the sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis (Fig. 1, lane 1).

2. Purification of the bifunctional enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase

Table 1. Purification of enoyl-CoA hydratase from n-alkane-grown Candida tropicalis pK 233.

Enzyme activity was measured by using crotonyl-CoA as substrate.

Ratio values mean 3-hydroxyacyl-CoA dehydrogenase activity/enoyl-CoA hydratase activity.

Fraction	Total protein (mg)	Total activity ($\mu mol.min^{-1}$)	Yield (%)	Specific activity ($\mu mol.min^{-1}.mg^{-1}$)	Purification (-fold)	Ratio
Cell-free extract	523	4660	100	8.91	1	80.7×10^{-4}
Hydroxyapatite	22.5	2070	44	92.0	10	6.52×10^{-4}
Cellulofine GCL-2000-m	8.90	1950	41	219	25	3.73×10^{-4}
DEAE-Sephacel	0.925	898	19	970	110	0

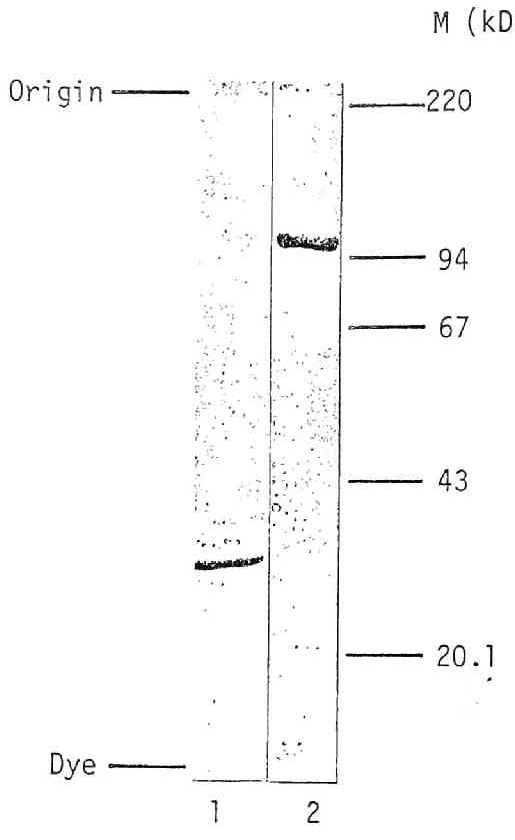


Fig. 1. Sodium dodecylsulfate/polyacrylamide slab-gel (10 %) electrophoresis of enoyl-CoA hydratase and the bifunctional enzyme.

Lane 1, enoyl-CoA hydratase (2 µg); lane 2, the bifunctional enzyme (2 µg). The marker proteins used were ferritin (M, 220 kDa), phosphorylase b (M, 94 kDa), bovine serum albumin (M, 67 kDa), ovalbumin (M, 43 kDa) and soybean trypsin inhibitor (M, 20.1 kDa).

The enzyme was purified using 3-hydroxylacyl-CoA dehydrogenase activity as a criteria. This enzyme seemed to be mostly localized in peroxisomal membrane. After many trials to solubilize the enzyme efficiently from the peroxisome-containing fraction (P_2) with detergents and to prepare the P_2-S fraction having a high activity of 3-hydroxyacy-CoA dehydrogenase, an amphiphilic detergent, Amphitol 20B(S) was found to be most effective. The enzyme was unstable even in the presence of dithiothreitol (1 mM), 2-mercaptoethanol (5 mM), EDTA (1 mM), glycerol (20 %, w/v), pepstatin A (50 μg/ml) or leupeptin (50 μg/ml) in a crude preparation, while phenylmethylsulfonyl fluoride (0.2 mM), antipain (50 μg/ml) or chymostatin (50 μg/ml) stabilized the enzyme. The latter substances did not affect the activity of enoyl-CoA hydratase present in the crude enzyme preparation. Based on these results, 3-hydroxyacyl-CoA dehydrogenase was solubilized from peroxisomes in a buffer containing phenylmethylsulfonyl fluoride (0.2 mM), antipain (50 μg/ml), chymostatin (50 μg/ml) and Amphitol (0.5 %, w/v) with stirring slowly at 4 °C for 18 h.

The supernatant (P_2-S) obtained by centrifugation at 127,000 x g for 1 h was applied to a DEAE-Sephacel column

(3.1 x 19 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 0.2 mM phenylmethylsulfonyl fluoride, washed with the same buffer and the buffer containing 0.2 mM phenylmethylsulfonyl fluoride and 0.27 M KCl, and the enzyme was eluted with a linear concentration gradient of KCl prepared from the buffers containing 0.27 M KCl and 0.40 M KCl. The enzyme solution exhibiting 3-hydroxyacyl-CoA dehydrogenase activity was concentrated, and applied to a Sephacryl S-300 column (2.2 x 60 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.2). The enzyme solution was further applied to a DEAE-Sepharose CL-6B column (1.8 x 15 cm) and the enzyme was eluted with the same method as that of DEAE-Sephacel column chromatography. The eluate exhibiting the enzyme activity was concentrated and used as a purified enzyme. The results of purification are summarized in Table 2.

Enoyl-CoA hydratase activity in the enzyme preparations was measured at every step of the purification. The ratio of 3-hydroxyacyl-CoA dehydrogenase activity to enoyl-CoA hydratase activity was almost constant throughout the purification (Table 2). These results indicated that the protein purified was a bifunctional enzyme having enoyl-CoA

Table 2. Purification of the bifunctional enzyme from n-alkane-grown Candida tropicalis pK 233.

The enzyme was purified as 3-hydroxyacyl-CoA dehydrogenase.

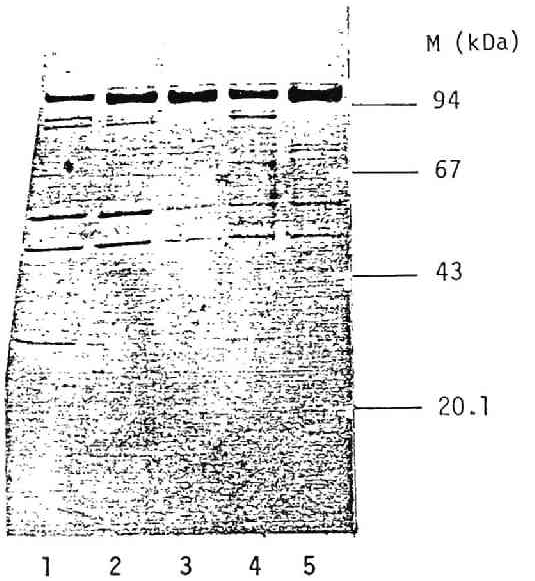
Ratio values are expressed as in Table 1.

Fraction	Total protein (mg)	Total activity ($\mu mol.min^{-1}$)	Yield (%)	Specific activity ($\mu mol.min^{-1}.mg^{-1}$)	Purification (-fold)	Ratio
P_2 fraction	805	593	100	0.737	1	5.39×10^{-2}
P_2-S fraction	532	412	69	0.774	1.1	6.09×10^{-2}
DEAE-Sephacel	9.36	120	20	12.8	17	4.04×10^{-2}
Sephacryl S-300	6.07	57.2	9.6	9.42	13	3.55×10^{-2}
DEAE-Sepharose CL-6B	3.23	37.5	6.3	11.6	16	4.61×10^{-2}

hydratase activity and 3-hydroxyacyl-CoA dehydrogenase activity. As shown in Fig. 1, the bifunctional enzyme was composed of a subunit with a molecular mass of 105 kDa.

3. Relationship between enoyl-CoA hydratase and the bifunctional enzyme

It is interesting whether enoyl-CoA hydratase subunit (36 kDa) is independent of the bifunctional enzyme subunit (105 kDa) or a derivative of the latter. When the bifunctional enzyme was treated with proteases for 70 min, Staphylococcus aureus V8 protease and papain gave little effect on enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, trypsin reduced 3-hydroxyacyl-CoA dehydrogenase activity to 40 % of the original activity, and α-chymotrypsin diminished completely 3-hydroxyacyl-CoA dehydrogenase activity without appreciable effect on enoyl-CoA hydratase activity (about 76 % of the original enoyl-CoA hydratase activity). On the sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis of the peptide fragments obtained after limited proteolysis, α-chymotrypsin and papain gave a peptide band corresponding to a molecular mass of 36 kDa (Fig. 2).



Protease used	ECH/HCD Activity (%)	
	ECH	HCD
1. α-Chymotrypsin	76	0
2. Trypsin	79	40
3. *S. aureus* V8 protease	75	116
4. Papain	88	120
5. None	100	100

(Enzyme : Protease = 100 : 1 , mol/mol)
(30°C , 70 min)

Fig. 2. Measurement of enzyme activities and detection of peptide fragments by sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis after limited proteolysis of the bifunctional enzyme by proteases.

Each reaction was carried out for 70 min at 30 °C (protease : substrate = 1 : 100, mol/mol) and stopped by the addition of protease inhibitors (chymostatin for α-chymotrypsin; antipain for trypsin and papain; phenylmethylsulfonyl fluoride for S. aureus V8 protease) (protease : inhibitor = 1 : 100, mol/mol). Five μg of the bifunctional enzyme was treated with α-chymotrypsin (lane 1), trypsin (lane 2), S. aureus V8 protease (lane 3), papain (lane 4) or without protease (lane 5).

A relationship between the activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase and appearance of a digested peptide fragment (36 kDa) were investigated during the incubation of the bifunctional enzyme with α-chymotrypsin (Fig. 3). 3-Hydroxyacyl-CoA dehydrogenase was highly susceptible to the protease reaction and the activity disappeared by 30 min, while enoyl-CoA hydratase activity kept the original level until 150 min of incubation. The fact that a 105 kDa peptide remained even after the complete loss of 3-hydroxyacyl-CoA dehydrogenase activity indicated strongly that 3-hydroxyacyl-CoA dehydrogenase domain of the bifunctional enzyme was very sensitive to the limited digestion with α-chymotrypsin in contrast to the resistivity of enoyl-CoA hydratase domain to the digestion. A peptide fragment corresponding to a molecular mass of 36 kDa began to appear at 30 min of incubation.

The 36 kDa fragment appeared after digestion with α-chymotrypsin was excised from the slab-gel and peptide maps obtained with S. aureus V8 protease and papain were compared with those of enoyl-CoA hydratase subunit (molecular mass, 36 kDa). The results shown in Fig. 4 demonstrated that the peptide maps of the 36 kDa fragment and

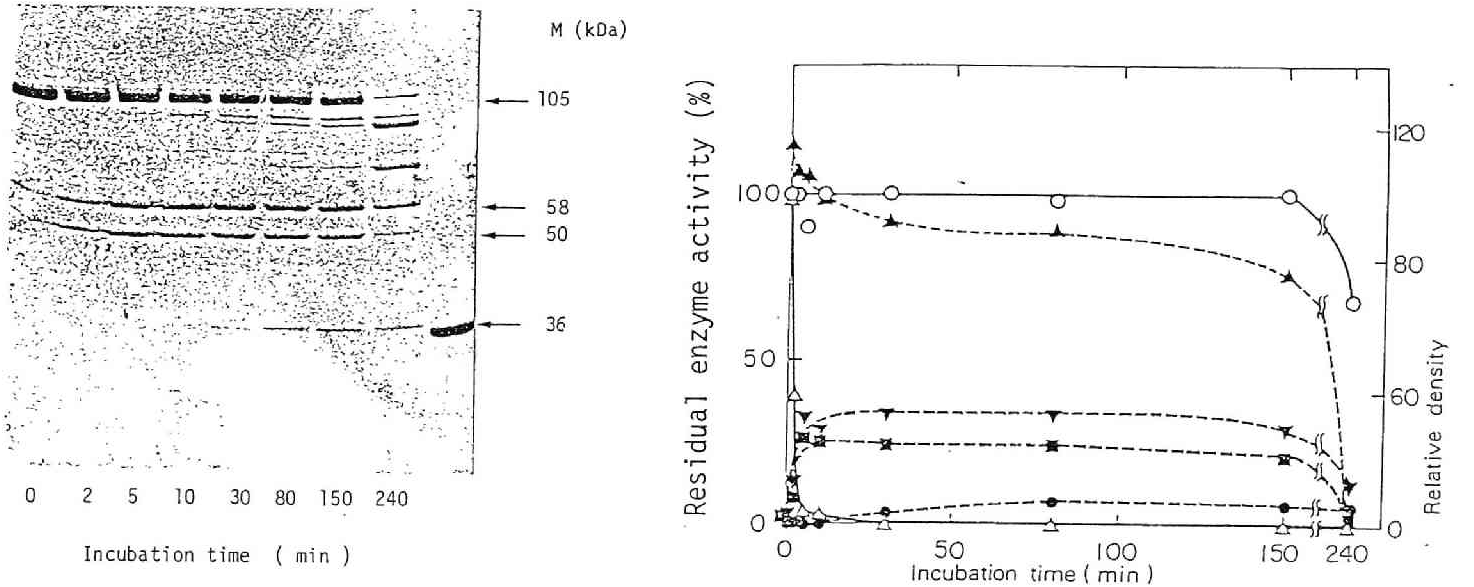


Fig. 3. Time-course of limited proteolysis of the bifunctional enzyme by α-chymotrypsin.

(A) The bifunctional enzyme (7 μg) was digested with α-chymotrypsin (protease : substrate = 1 : 100, mol/mol) and the reaction was stopped by the addition of chymostatin (protease : inhibitor = 1 : 200, mol/mol). Peptides obtained were analyzed by sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis. (B) Time-course changes in the activities of enoyl-CoA hydratase (○) and 3-hydroxyacyl-CoA dehydrogenase (△), and the densities of 105 kDa peptide (▲), 58 kDa peptide (▼), 50 kDa peptide (■) and 36 kDa peptide (●). The densities of the peptides on the electrophoresed slab-gel (A) were measured with a scanning densitometer.

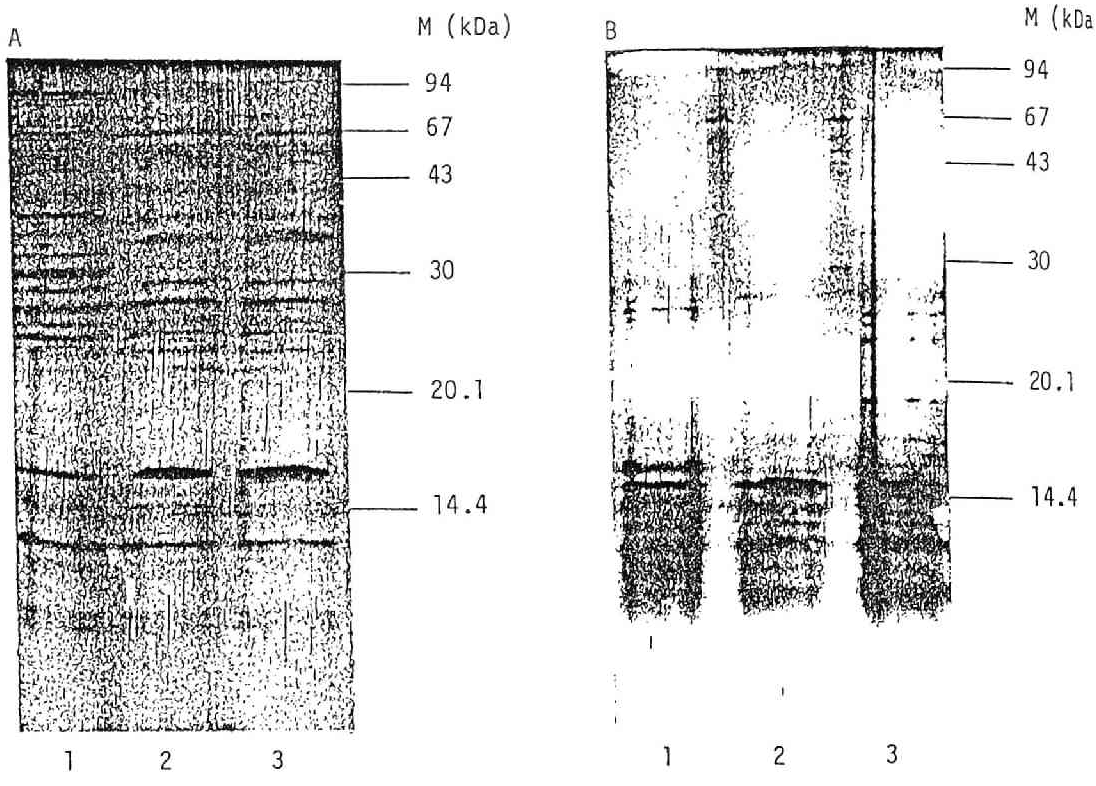


Fig. 4. Limited proteolysis of the bifunctional enzyme, enoyl-CoA hydratase and the 36 kDa fragment derived from the bifunctional enzyme by α-chymotrypsin treatment with S. aureus V8 protease and papain on sodium dodecyl sulfate/polyacrylamide slab-gel (12.5 %) electrophoresis.

Peptides were stained with silver staining reagents. (A) Peptide maps of the respective enzymes (ca. 2 μg) obtained with S. aureus V8 protease (12.5 ng). (B) Peptide maps of the respective enzymes (ca. 2 μg) obtained with papain (6 ng). 1, The bifunctional enzyme; 2, enoyl-CoA hydratase; 3, the 36 kDa fragment.

enoyl-CoA hydratase subunit were indistinguishable, indicating that enoyl-CoA hydratase with 36 kDa subunits purified from whole cells of n-alkane-grown C. tropicalis was a peptide hydrolyzed from the bifunctional enzyme with an α-chymotrypsin-like protease in the cells during the enzyme purification.

4. Comparison of some properties of enoyl-CoA hydratase and the bifunctional enzyme

The difference in the subunit molecular masses of the respective enzymes was already described. The determination of molecular masses by Sepharose 6B column chromatography showed that the active bifunctional enzyme was monomeric with a molecular mass of 110 kDa and that enoyl-CoA hydratase was tetrameric with a molecular mass of 144 kDa composed of subunits with a molecular mass of 36 kDa (Fig. 5). As for enoyl-CoA hydratase activity, the specific activity of enoyl-CoA hydratase was about four times higher than that of the bifunctional enzyme (Table 3).

Noticeable differences were not observed between enoyl-CoA hydratase and the bifunctional enzyme in substrate specificity, optimum pH and Km values for crotonyl-CoA and

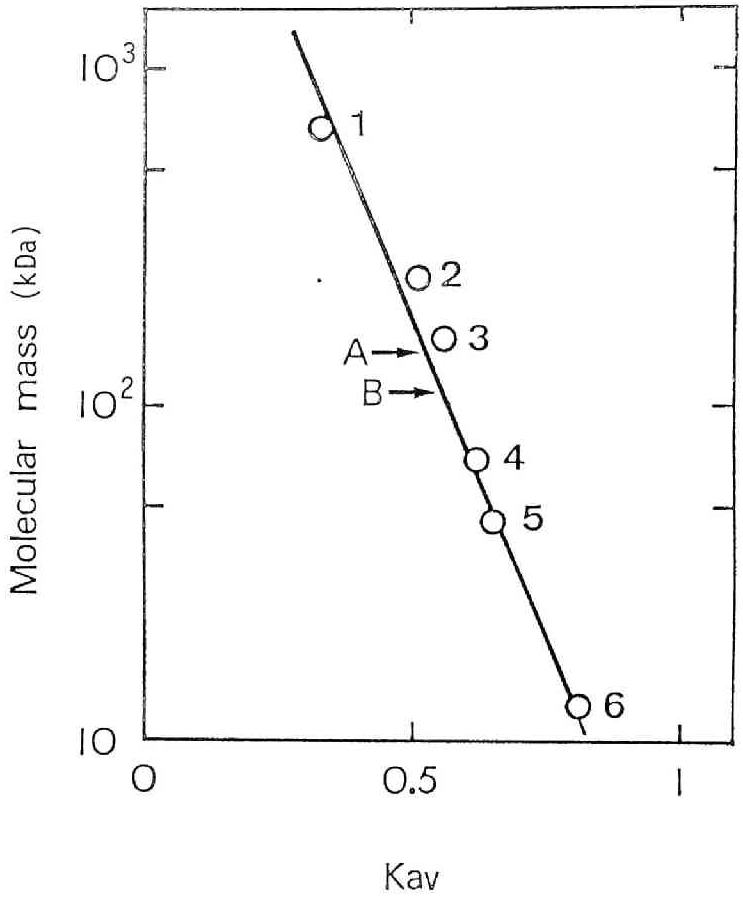


Fig. 5. Estimation of molecular masses of the active forms of enoyl-CoA hydratase and the bifunctional enzyme.

The arrows indicate the elution points of enoyl-CoA hydratase (A) and the bifunctional enzyme (B) on a Sepharose 6B column. The following proteins were used as markers: 1, Thyroglobulin (M, 669 kDa); 2, catalase (M, 232 kDa); 3, aldolase (M, 158 kDa); 4, bovine serum albumin (M, 68 kDa); 5, ovalbumin (M, 45 kDa); and 6, cytochrome c (M, 12 kDa).

Table 3. Properties of enoyl-CoA hydratase and the bifunctional enzyme

Property	Enoyl-CoA hydratase	Bifunctional enzyme	
		Enoyl-CoA hydratase	3-Hydroxyacyl-CoA dehydrogenase
Molecular mass (kDa)			
Subunit	36	105	
Native enzyme	144	110	
Subunit number	4	1	
Specific activity[a] ($\mu mol.min^{-1}.mg^{-1}$)	970	251	11.6
Optimum reaction pH	6.0	6.0	7.4
Substrate specificity[b]	C_4 C_{18} (max. C_{12})	C_4 C_{18} (max. C_{12})	NT[c]
Km value (μM) for			
Crotonyl-CoA	190	190	—
2-Dodecenoyl-CoA[b]	400	400	—
Acetoacetyl-CoA	—	—	23
NADH	—	—	32

[a] Crotonyl-CoA was used as substrate in measuring enoyl-CoA hydratase activity.

[b] Measurement was performed by using acyl-CoAs and acyl-CoA oxidase as described in Part I, Chapter 1.

[c] Not tested.

2-dodecenoyl-CoA. The optimum pH of 3-hydroxyacyl-CoA dehydrogenase of the bifunctional enzyme was 7.4 and Km values for acetoacetyl-CoA and NADH were 23 μM and 32 μM, respectively.

DISCUSSION

In Escherichia coli, fatty acid β-oxidation except for the step of acyl-CoA dehydrogenation was carried out by a multifunctional enzyme composed of $\alpha_2\beta_2$ subunits. Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase domains were located on the larger subunit (9). This multifunctional enzyme was considered not to be separable into individual enzyme domains, although a mutant defective in 3-hydroxyacyl-CoA dehydrogenase was obtained (10). In mammalian cells, the peroxisomal bifunctional and mitochondrial separate enzymes were immunochemically distinguishable each other (11) and different mRNAs corresponded to each enzyme (12).

From an n-alkane-assimilating yeast, Candida tropicalis, the enzyme composed of 36 kDa subunits with only enoyl-CoA hydratase activity was purified. This enzyme

was proved to be a derivative from the bifunctional enzyme with a molecular mass of 105 kDa, while Moreno de la Garza et al. (5) reported that the multifunctional enzyme from oleate-grown C. tropicalis was a dimer composed of the subunits with a molecular mass of 102 kDa. Our results suggested the possible existence of an enoyl-CoA hydratase domain apart from a 3-hydroxyacyl-CoA dehydrogenase domain on a single peptide. Furthermore, active enoyl-CoA hydratase was demonstrated to form a tetramer with a subunit molecular mass of 36 kDa as the case of mammalian mitochondrial enoyl-CoA hydratase composed of six subunits with a molecular mass of 26 kDa (12).

It remains, however, whether or not a single domain of 3-hydroxyacyl-CoA dehydrogenase is present, whether or not 3-hydroxyacyl-CoA dehydrogenase activity requires the enoyl-CoA hydratase domain to express its complete activity, and whether or not 3-hydroxyacyl-CoA epimerase reported by Moreno de la Garza et al. (5) is included in the bifunctional enzyme from alkane-grown C. tropicalis. In spite of these uncertain properties, the investigation of the enzymes from this yeast would give us some clue on the genetic evolution of yeast peroxisomes and the peroxisomal

β-oxidation system.

SUMMARY

A protein having only enoyl-CoA hydratase activity was purified from an n-alkane-utilizing yeast, Candida tropicalis. This enzyme had a homotetrameric form composed of the subunits with a molecular mass of 36 kDa. On the other hand, a bifunctional enzyme having enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities was prepared from the same yeast cells when purified in the presence of protease inhibitors, phenylmethylsulfonyl fluoride, antipain and chymostatin. The enzyme had a molecular mass of 105 kDa and was a monomeric form. Limited proteolysis of the bifunctional enzyme with α-chymotrypsin yielded a peptide mixture containing a 36 kDa fragment, the mixture showing about 76 % of the original enoyl-CoA hydratase activity but no activity of 3-hydroxyacyl-CoA dehydrogenase. Comparison of peptide maps between the purified enoyl-CoA hydratase and the 36 kDa fragment obtained from the bifunctional enzyme showed a similarity of these proteins. These results indicated that the domain of

enoyl-CoA hydratase activity was separable from the bifunctional enzyme by the action of a certain protease.

REFERENCES

(1) Lazarow,P.B. & de Duve,C. (1976) Proc. Natl. Acad. Sci. USA 73, 2043.

(2) Osumi,T. & Hashimoto,T. (1979) Biochem. Biophys. Res. Commun. 89, 580.

(3) Osumi,T. & Hashimoto,T. (1980) Arch. Biochem. Biophys. 203, 372.

(4) Frevert,J. & Kindl,H. (1980) Eur. J. Biochem. 107, 79.

(5) Moreno de la Garza,M., Schultz-Borchard,U., Crabb,J.W. & Kunau,W.H. (1985) Eur. J. Biochem. 148, 285.

(6) Tanaka,A., Yamamura,M., Kawamoto,S. & Fukui,S. (1977) Appl. Environ. Microbiol. 34, 342.

(7) King,J. & Laemmli,U.K. (1971) J. Mol. Biol. 62, 465.

(8) Cleveland,D.W., Fischner,S.G., Kirschner,M.W. & Laemmli,U.K. (1977) J. Biol. Chem. 252, 1102.

(9) Yang,S.Y., Bittman,R. & Schulz,H. (1985) J. Biol. Chem. 260, 2862.

(10) Pramanik,A. & Schulz,H. (1983) Biochim. Biophys. Acta 750, 41.

(11) Furuta,S., Miyazawa,S., Osumi,T., Hashimoto,T. & Ui,N. (1980) J. Biochem. 88, 1059.

(12) Osumi,T., Ishii,N., Hijikata,M., Kamijo,K., Ozasa,H., Furuta,S., Miyazawa,S., Kondo,K., Inoue,K., Kagamiyama, H. & Hashimoto,T. (1985) J. Biol. Chem. 260, 8905.

Part II Glyoxylate cycle enzymes, isocitrate lyase and malate synthase, of n-alkane-utilizing Candida tropicalis

Chapter 1. Properties of isocitrate lyase from an alkane-utilizable yeast, Candida tropicalis

INTRODUCTION

n-alkanes induce the appearance of abundant peroxisomes in Candida tropicalis cells (1,2). Professor Fukui's group have demonstrated that peroxisomes of alkane-grown C. tropicalis contain the enzymes that participate in fatty acid β-oxidation and the key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase), and that they are indispensable for the alkane assimilation by the yeast (3).

Hitherto, isocitrate lyase (EC 4.1.3.1), one of the key enzymes of the glyoxylate cycle, has been isolated from Pseudomonas indigofera (4), Neurospora crassa (5), Turbatrix aceti (6), Chlorella pyrenoidosa (7),

Cucumis sativus (8), Linum usitatissium (flax seedling) (9) and so on. Chlorella vulgaris (10), N. crassa (11) and Pseudomonas MA (Shaw strain) (12) seem to have two kinds of isocitrate lyases. Nevertheless, little information is available on the properties of these distinct types of enzymes.

In C. tropicalis, the activity level of isocitrate lyase in alkane-grown cells was considerably higher than that in glucose-grown cells (13). Most part of the enzyme was localized in peroxisomes when the yeast was grown on alkanes (14). A large part of the constitutive enzyme in glucose-grown cells was also found in the particulate fraction, although its localization in pre-existing peroxisomes has not been examined. These facts show that isocitrate lyase could be of use as a target enzyme for investigating the biogenesis and development of yeast peroxisomes through the mechanism of enzyme localization in the organelles.

This chapter describes the purification and comparison of several properties of isocitrate lyases from a peroxisome-containing fraction of alkane-grown cells and from whole cells of glucose-grown yeast. The kinetic properties

of the partially purified enzymes were reported previously (15).

MATERIALS AND METHODS

1. Cultivation of yeast

Cultivation of Candida tropicalis pK 233 (ATCC 20336) were carried out as in Chapter 1 of Part I.

2. Measurement of isocitrate lyase activity

Isocitrate lyase activity was measured according to the method of Dixon and Kornberg (16) with slight modifications. The assay mixture contained 100 mM potassium phosphate buffer (pH 7.2), 75 mM $MgCl_2$, 50 mM phenylhydrazine-HCl, 30 mM cysteine-HCl, 125 mM sodium D,L-isocitrate and enzyme source in a final volume of 1.5 ml. Glyoxylate-phenylhydrazone formed was measured by following the increase in absorbance at 324 nm.

3. Purification of isocitrate lyase

Isocitrate lyase was purified from the peroxisome-containing particulate fraction of alkane-grown cells and

from whole cells of the glucose-grown yeast. All steps except for the protoplast preparation were carried out at 0-4 °C.

A) Purification of peroxisomal isocitrate lyase (P-ICL): subcellular fractionation of yeast cells (65 g wet cells) grown on alkanes was carried out as described in Chapter 2 of Part I. The particulate fraction (20,000 x g pellets) containing peroxisomes and mitochondria was suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.2) to disintegrate the organelles. This suspension was centrifuged at 127,000 x g for 1 h, and the resulting supernatant (crude enzyme) was used as the source of P-ICL. The supernatant was applied to a DEAE-Sephacel column (3.4 x 20 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2). Elution was initiated with 10 mM potassium phosphate buffer (pH 7.2) (240 ml) followed by a linear concentration gradient of KCl prepared from 300 ml of 10 mM potassium phosphate buffer (pH 7.2) and 300 ml of the same buffer containing 0.3 M KCl, and then by 140 ml of the same buffer containing 1 M KCl. Fractions showing enzyme activity (No. 8 - 11, 24 ml) were collected and concentrated to 2.0 ml with a Diaflo-membrane filter PM-10 (Amicon Far

East, Tokyo, Japan). The concentrated enzyme solution was applied to a Sepharose 6B column (2.2 x 80 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.2). Active fractions (No. 58 - 68, 66 ml) eluted with the same buffer were collected and concentrated to 1.2 ml as described above. This preparation was used as the purified P-ICL.

B) Purification of isocitrate lyase from glucose-grown cells (G-ICL): yeast cells grown on glucose (61 g wet cells) were suspended in 60 ml of 50 mM potassium phosphate buffer (pH 7.2) and then disrupted with a Braun cell homogenizer (150 s for ca. 15 g wet cells). After the cell debris had been removed by centrifugation (127,000 x g for 1 h), the supernatant obtained (cell-free extract) was applied to a DEAE-Sephacel column (No. 12 - 14, 24 ml) followed by Sepharose CL-6B column chromatography (No. 36 - 42, 28 ml) under the same conditions as described above. The concentrated enzyme solution (2.4 ml) was further applied to a CM-Sepharose CL-6B column (2.2 x 10 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2). Chromatography was carried out with 10 mM potassium phosphate buffer (pH 7.2) followed by a linear concentration gradient of KCl prepared from 50 ml of 10 mM potassium

phosphate buffer (pH 7.2) and 50 ml of the same buffer containing 0.3 M KCl, and then with the buffer containing 1 M KCl. The enzyme fractions (No. 4 - 6, 9 ml) eluted with 10 mM potassium phosphate buffer (pH 7.2) were pooled and concentrated to 1.4 ml as described above, and then used as the purified G-ICL preparation.

4. Molecular mass estimation

Estimation of the molecular masses of the active forms of the enzymes was carried out by Sepharose 6B gel-filtration chromatography, and the molecular masses of the subunit forms were determined by sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis as described in Chapter 2 of Part I.

5. Limited proteolysis of isocitrate lyase subunits

After each isocitrate lyase had been dissociated into subunits on 10 % polyacrylamide slab-gels in the presence of sodium dodecylsulfate, the gel areas corresponding to the subunits were excised and electrophoresed with proteases as described in Chapter 2 of Part I, except that 12.5 % acrylamide gels were used as the digesting gels. The experi-

mental conditions are given in the legend to Fig. 3. Peptides were detected by the silver staining method.

6. Amino acid composition analysis

The purified preparations of both enzymes were extensively dialyzed against distilled water, and then lyophilized. The lyophilized samples were hydrolyzed at 110 °C for 20 h with 6 M HCl in sealed tubes *in vacuo*. Amino acids in the hydrolyzates were analyzed with a Hitachi 835 amino acid analyzer, according to the method of Spackman *et al.* (17). The amount of each amino acid was corrected based on the recovery of the internal standard. Half-cystine was determined by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (18) and tryptophan was assayed by the method of Edelhoch (19). Amino acid composition was computed from the average values of the duplicate experiments.

7. Immunochemical procedures

Rabbit antiserum against purified P-ICL was prepared in the following manner. A solution of the purified enzyme (1.75 mg protein) was mixed with an equal volume of complete

Freund's adjuvant (Difco, Detroit, USA). This mixture was injected into the footpads of each rabbit. Four weeks later, 1.5 mg of the purified enzyme was injected subcutaneously as a booster. Blood was obtained from vein of the ears of immunized animals 5-7 days after the booster injection and the serum was prepared by means of centrifugation (20). For immunochemical titration, different amounts of the anti-(P-ICL) antiserum were reacted with a fixed amount of each antigen. Each antigen and antiserum mixture was kept at 0 °C for 3 h and then centrifuged at 20,000 x g for 15 min, and the supernatant was assayed for the enzyme activity. Immunochemical analysis was performed similarly with a fixed amount of the antiserum and different amounts of each antigen.

RESULTS

1. Purification of isocitrate lyase from the peroxisome-containing fraction of alkane-grown cells and from glucose-grown whole cells

The particulate fraction containing peroxisomes and mitochondria was used as the source of peroxisomal isocit-

Table 1. Purification of P-ICL from the peroxisome-containing fraction of alkane-grown Candida tropicalis

Fraction	Total protein (mg)	Specific activity ($\mu mol.min^{-1}.mg\ protein^{-1}$)	Total activity ($\mu mol.min^{-1}$)	Purification (fold)	Yield (%)
Crude enzyme	28.1	0.712	20.0	1	100
DEAE-Sephacel eluate	1.47	5.58	8.21	7.8	41
Sepharose 6B eluate	0.33	10.9	3.60	15.3	18

Table 2. Purification of G-ICL from glucose-grown Candida tropicalis

Fraction	Total protein (mg)	Specific activity ($\mu mol.min^{-1}.mg\ protein^{-1}$)	Total activity ($\mu mol.min^{-1}$)	Purification (fold)	Yield (%)
Cell-free extract	190	0.161	30.6	1	100
DEAE-Sephacel eluate	2.0	5.70	11.4	35	37
Sepharose CL-6B eluate	0.60	6.33	3.80	39	12
CM-Sepharose CL-6B eluate	0.24	8.75	2.10	54	6.9

rate lyase (P-ICL) because isocitrate lyase is localized in peroxisomes and not in mitochondria of alkane-grown Candida tropicalis cells. The enzyme was easily purified by a combination of chromatographies on DEAE-Sephacel and Sephrose 6B columns.

The cell-free extract of glucose-grown cells was chromatographed on DEAE-Sephacel, Sepharose CL-6B and CM-Sepharose CL-6B columns, successively. The purified enzyme (G-ICL) was obtained after CM-Sepharose CL-6B column chromatography. Although isocitrate lyase was distributed between the particulate and cytosolic fractions of glucose-grown cells, only one type of enzyme could be detected during the purification of G-ICL from the cell-free extract.

The homogeneity of P-ICL and G-ICL was confirmed by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecylsulfate, as described later. The results of the purification procedures for the enzymes are summarized in Tables 1 and 2, respectively. Both enzymes were stable at -20 °C for at least three months.

2. Molecular mass

The active forms of both enzymes were eluted in a

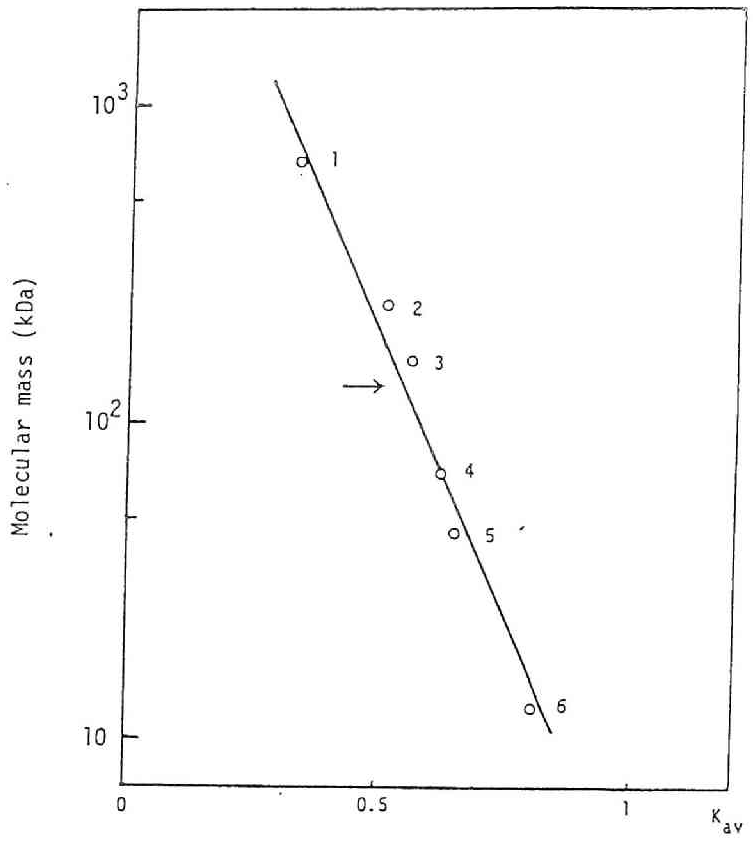


Fig. 1. Estimation of the molecular masses of active forms of isocitrate lyase

The arrow indicates the elution points of P-ICL on a Sepharose 6B column. The following proteins were used as markers: 1, thyroglobulin (M, 669 kDa); 2, catalase (M, 232 kDa); 3, aldolase (M, 158 kDa); 4, bovine serum albumin (M, 68 kDa); 5, ovalbumin (M, 45 kDa); and 6, cytochrome c (M, 12.5 kDa).

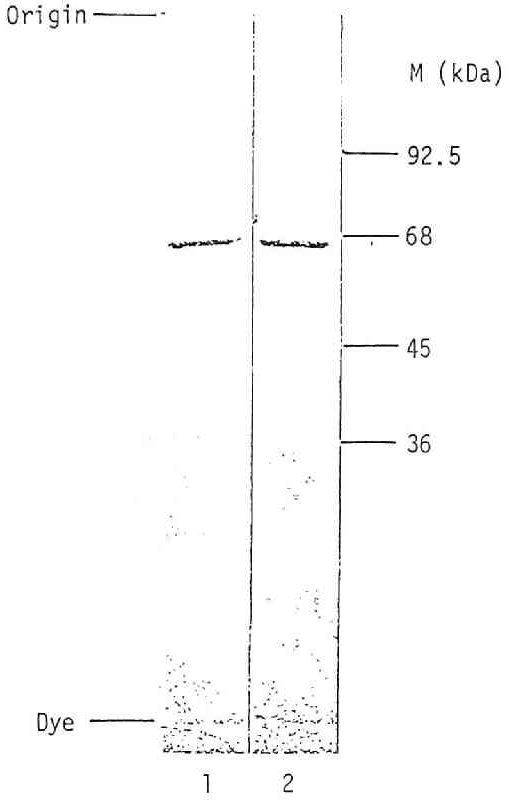


Fig. 2. Sodium dodecylsulfate/polyacryamide slab-gel (10 %) electrophoresis of P-ICL and G-ICL.

Lane 1, P-ICL (2 µg); lane 2, G-ICL (2 µg). The marker proteins used were phosphorylase b (M, 92.5 kDa), bovine serum albumin (M, 68 kDa), ovalbumin (M, 45 kDa) and lactate dehydrogenase (M, 36 kDa).

single peak on gel-filtration with Sepharose 6B and their molecular masses were both estimated to be approximately 130 kDa (Fig. 1). On polyacrylamide slab-gel electrophoresis in the presence of sodium dodecylsulfate, each isocitrate lyase gave a single peptide band whose molecular mass was caluculated to be 65 kDa (Fig. 2). These results indicate that P-ICL is a homo-dimer with the same molecular mass as G-ICL.

3. Limited proteolysis

To compare P-ICL with G-ICL, their digested peptides were prepared by using Staphylococcus aureus V8 protease and papain, respectively. Similar peptide maps were obtained after limited proteolysis with the respective proteases as shown in Fig. 3A and B.

4. Amino acid composition

The results of amino acid analysis of each enzyme are summarized in Table 3. The amino acid compositions of the two enzymes were almost the same with slight differences in the contents of several amino acids, such as alanine, arginine, isoleucine and tyrosine.

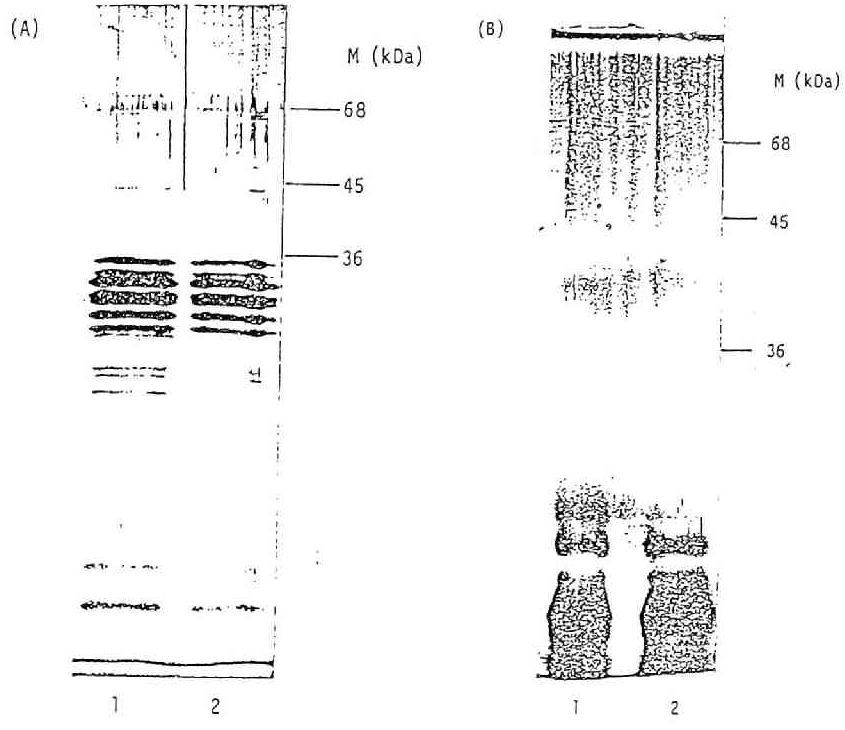


Fig. 3. Limited proteolysis of P-ICL and G-ICL with Staphylococcus aureus V8 protease (A) and papain (Papaya latex protease) (B) on sodium dodecylsulfate/polyacrylamide slab-gel (12.5 %) electrophoresis.

Peptides were stained with silver staining reagents. (A): Peptide maps of the respective enzymes (8 µg) with S. aureus V8 protease (80 ng). (B): Peptide maps of the respective enzymes (6 µg) with papain (13 ng). 1, P-ICL; 2, G-ICL.

Table 3. Amino acid compositions of P-ICL and G-ICL

The contents of cysteine and tryptophan were determined spectrophotometrically as described in "MATERIALS AND METHODS".

Amino acid	P-isocitrate lyase	G-isocitrate lyase
	(mol/100 mol)	
Alanine	11.9	13.0
Arginine	4.5	3.4
Aspartic acid	9.7	10.2
Cysteine	0.3	0.3
Cystine	0.4	0.9
Glutamic acid	11.8	11.9
Glycine	7.1	7.5
Histidine	2.6	2.6
Isoleucine	6.0	4.8
Leucine	7.0	6.8
Lysine	9.0	8.9
Methionine	1.9	1.8
Phenylalanine	3.6	3.3
Proline	3.6	4.1
Serine	4.9	5.6
Threonine	5.5	6.4
Tryptophan	1.7	1.8
Tyrosine	3.8	2.0
Valine	4.7	4.8

5. Immunochemical properties

The immunochemical properties of P-ICL and G-ICL were examined by using an antiserum against P-ICL. Figures 4 and 5 present the results of immunochemical titration and immunochemical analysis, respectively. The results of immunochemical titration indicated that both enzymes were completely precipitated by the anti-(P-ICL) antiserum in a similar manner. Moreover, the results of immunochemical analysis showed that the equivalent points of both enzymes as to the prepared antiserum were the same. These results showed that G-ICL was immunochemically indistinguishable from P-ICL.

DISCUSSION

Professor Fukui's group have proved that the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, are localized in peroxisomes, which appear profusely in Candida tropicalis cells grown on alkanes, but not in mitochondria (3). The level of isocitrate lyase activity in alkane-grown cells is several times higher than

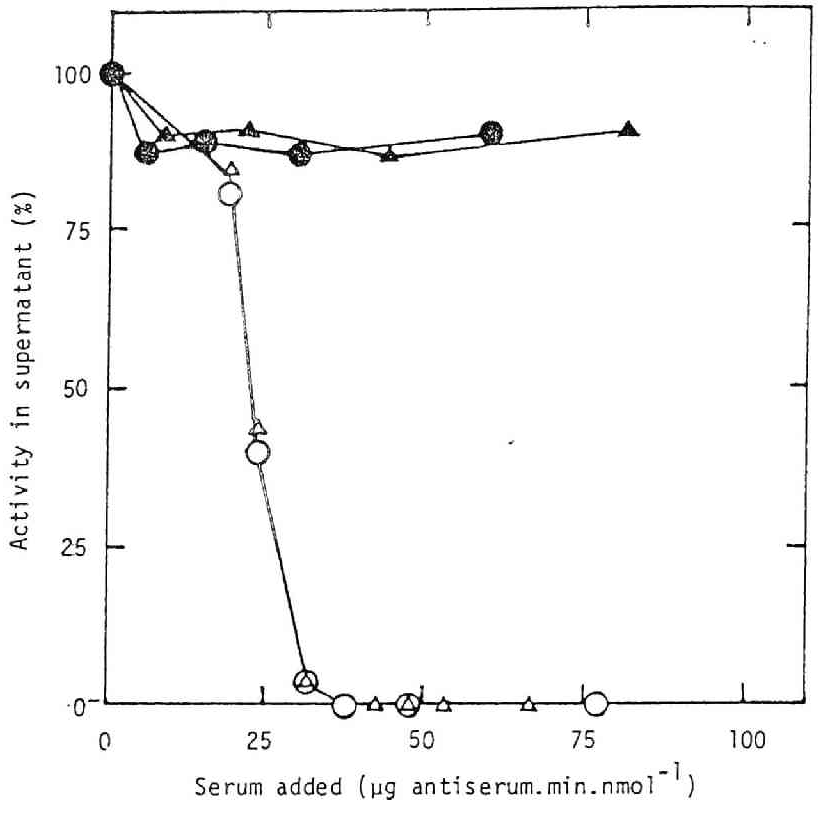


Fig. 4. Immunochemical titration of P-ICL and G-ICL with anti-(P-ICL) antiserum.

Fixed amounts of P-ICL (5.0 μg) and G-ICL (5.9 μg) were titrated with the antiserum. (○,△), Anti-(P-ICL) antiserum; (●,▲), nonimmunized serum. (△,▲), Reacted with P-ICL; (○,●), reacted with G-ICL.

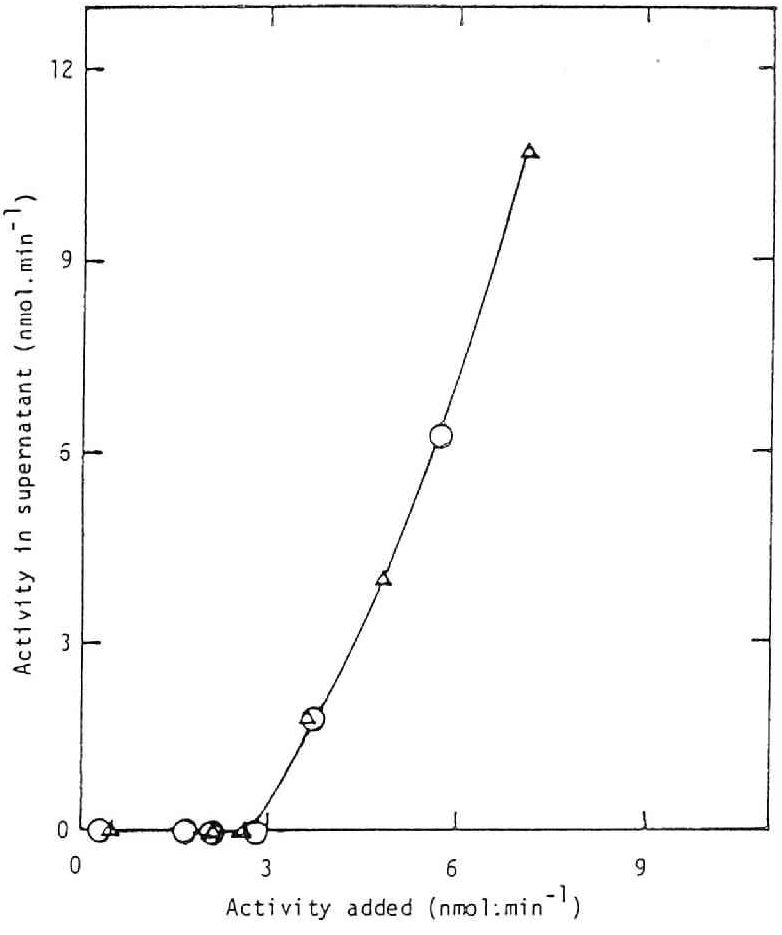


Fig. 5. Immunochemical analysis of P-ICL and G-ICL with anti-(P-ICL) antiserum.

A fixed amount of the antiserum (108 μg) was reacted with different amounts of P-ICL (△) of G-ICL (○).

that in glucose-grown cells which contain only a few peroxisomes. The results obtained hitherto indicate that isocitrate lyase in glucose-grown cells is a constitutive enzyme, while the enzyme is induced and localized predominantly in peroxisomes when the yeast is grown on alkanes. Therefore, it is reasonable to select isocitrate lyase as a target enzyme to investigate the development of peroxisomes. For this purpose, it is necessary to purify and characterize isocitrate lyase from peroxisomes of alkane-grown cells and that from glucose-grown cells.

Isocitrate lyase purified to homogeneity from the peroxisome-containing fraction of alkane-grown cells (P-ICL) and the enzyme from glucose-grown whole cells (G-ICL) have the same molecular mass of 130 kDa (estimated by gel chromatography) and are both composed of two identical subunits of 65 kDa. On the other hand, the enzymes from Neurospora crassa (5), Pseudomonas indigofera (4), Turbatrix aceti (6), Cucumis sativas (8) and flax seedling (9) have been reported to be tetrameric, although N. crassa also has a stable dimeric species of the enzyme whose molecular mass is 130 kDa (21). Chlorella vulgaris (10) and N. crassa (11) have been found to have two kinds

of isocitrate lyases; one is induced during growth on acetate and the other appears during growth on glucose. Pseudomonas MA (Shaw strain) also possesses two activities; one appears during growth on acetate and the other during growth on methylamine (12). However these isoenzymes have not been studied in detail.

Frevert and Kindl (8) reported that isocitrate lyase from cucumber had a glycoprotein nature. The fact that the development of a brown color was observed during the hydrolysis of G-ICL, but not that of P-ICL, might indicate the presence of some modifying groups (carbohydrate moieties etc.) covalently bound to G-ICL. However, the content of carbohydrate, if present, in the enzyme might be very low, because the limited proteolysis of G-ICL gave a similar peptide map to that of P-ICL. At present, no information is available on the kind and content of carbohydrate moieties in G-ICL.

Although it was found to be a slight difference in the amino acid compositions and so on between P-ICL and G-ICL, no difference was observed in the peptide maps or immunochemical properties. The results of immunochemical studies suggest that both enzymes are products of the same gene.

SUMMARY

Isocitrate lyase (EC 4.1.3.1) was purified from the peroxisome-containing particulate fraction of an alkane-grown yeast, Candida tropicalis, which had a conspicuous number of peroxisomes. The properties of the isocitrate lyase, which was induced by alkanes and localized in peroxisomes, were compared with those of the constitutive enzyme purified from glucose-grown cells of the yeast, which contained only a few pre-existing peroxisomes. The molecular mass of both enzymes was estimated to be about 130 kDa by gel-filtration chromatography, and they were both composed of two identical subunits of a molecular mass of 65 kDa. Both enzymes showed similar peptide maps upon partial digestion with proteolytic enzymes and were indistinguishable immunochemically, although there was a slight difference in their amino acid compositions.

REFERENCES

(1) Osumi,M., Miwa,N., Teranishi,Y., Tanaka,A. & Fukui,S. (1974) Arch. Microbiol. 99, 181.

(2) Osumi,M., Fukuzumi,F., Teranishi,Y., Tanaka,A. & Fukui,S. (1975) Arch. Microbiol. 103, 1.

(3) Tanaka,A., Osumi,M. & Fukui,S. (1982) Ann. N. Y. Acad. Sci. 386, 183.

(4) McFadden,B.A., Rao,G.R., Cohen,A.L. & Roche,T.E. (1968) Biochemistry 7, 3574.

(5) Johanson,R.A., Hill,J.M. & McFadden,B.A. (1974) Biochim. Biophys. Acta 364, 327.

(6) Reiss,U. & Rothstein,M. (1974) Biochemistry 13, 1796.

(7) John,P.C.L. & Syrett,P.J. (1967) Biochem. J. 105, 409.

(8) Frevert,J. & Kindl,H. (1978) Eur. J. Biochem. 92, 35.

(9) Khan,F.R., Saleemuddin,M., Siddiqi,M. & McFadden,B.A. (1977) Arch. Biochem. Biophys. 183, 13.

(10) Hillier,S. & Charnetzky,W.T. (1981) J. Bacteriol. 145, 452.

(11) Sjogren,R.E. & Romano,A.H. (1967) J. Bacteriol. 93, 1638.

(12) Bellion,E. & Woodson,J. (1975) J. Bacteriol. 122,

557.

(13) Nabeshima,S., Tanaka,A. & Fukui,S. (1977) Agric. Biol. Chem. 41, 275.

(14) Kawamoto,S., Tanaka,A., Yamamura,M., Teranishi,Y., Fukui,S. & Osumi,M. (1977) Arch. Microbiol. 112, 1.

(15) Tanaka,A., Nabeshima,S., Tokuda,M. & Fukui,S. (1977) Agric. Biol. Chem. 41, 795.

(16) Dixon,G.H. & Kornberg,H.L. (1959) Biochem. J. 72, 3.

(17) Spackman,D.H., Stein,W.H. & Moore,S. (1958) Anal. Chem. 30, 1190.

(18) Ellman,G.L. (1959) Arch. Biochem. Biophys. 82, 70.

(19) Edelhoch,H. (1967) Biochemistry 6, 1948.

(20) Ueda,M., Tanaka,A. & Fukui,S. (1984) Eur. J. Biochem. 138, 445.

(21) Johanson,R.A., Hill,J.M. & McFadden,B.A. (1974) Biochim. Biophys. Acta 364, 341.

Chapter 2. Purification of peroxisomal malate synthase from alkane-grown Candida tropicalis and some properties of the purified enzyme

INTRODUCTION

Malate synthase has been investigated as one of the key enzymes in the glyoxylate cycle, which has an important function in gluconeogenesis from storage lipids in germinating seeds (1). The enzyme from seeds was demonstrated to be localized in the glyoxysomal membrane fraction (2). Furthermore, the glyoxylate cycle has been detected in yeasts such as Candida utilis and Hansenula polymorpha (3), toad urinary bladder (4), liver of fetal guinea pig (5) and other tissues, this suggesting an important role of the cycle in eukaryotic cells.

The n-alkane-assimilating yeast, Candida tropicalis, contains a conspicuous number of peroxisomes (microbodies) when grown on alkanes or higher fatty acids, the organelles playing an indispensable role in the metabolism of these compounds (6,7).

The present chapter deals with the purification of

malate synthase from peroxisomes of alkane-grown C. tropicalis together with some properties and the intra-peroxisomal localization of this enzyme. The significance of the enzyme in the metabolism of alkanes by the yeast is also discussed.

MATERIALS AND METHODS

1. Cultivation of yeast

Cultivation of Candida tropicalis pK 233 (ATCC 20336) were carried out as in Chapter 1 of Part I.

2. Subcellular fractionation

Preparation of yeast protoplasts, subcellular fractionation by differential centrifugation, and isolation of peroxisomes by discontinuous sucrose density gradient centrifugation were carried out as in Chapter 1 of Part I.

3. Purification of enzyme

A particulate fraction containing peroxisomes and mitochondria (20,000 x g pellets from the protoplast homogenate) was obtained from 160 g wet cells and suspended in

30 ml of 50 mM potassium phosphate buffer (pH 7.2) to solubilize malate synthase. The suspension was stirred well and then centrifuged at 127,000 x g for 1 h to obtain a supernatant containing the enzyme (12). Subsequent steps for the enzyme purification are described under "Results".

4. Determination of molecular mass

Determination of molecular mass of the native form and the subunit of the enzyme was carried out as in Chapter 2 of Part I.

5. Enzyme assay

Malate synthase activity was measured by following the decrease in absorbance of acetyl-CoA at 232 nm according to the method of Dixon and Kornberg (13) with slight modifications. The assay mixture was composed of 25 mM Tris-HCl buffer (pH 8.0), 50 mM $MgCl_2$, 1 mM acetyl-CoA, 10 mM sodium glyoxylate and enzyme source in a final volume of 1.5 ml (14). Protein was quantified according to Lowry et al. (15).

6. Chemicals

Acetyl-CoA and sodium glyoxylate were obtained from Sigma (St. Louis, MO, USA). DEAE-Sephacel and Sepharose 6B were purchased from Pharmacia (Uppsala, Sweden). Other chemicals were also obtained from commercial sources.

RESULTS

1. Intraperoxisomal localization of malate synthase

To investigate the intraperoxisomal localization of malate synthase, peroxisomes purified by discontinuous sucrose density gradient centrifugation were subjected to dialysis against a hypotonic buffer, 50 mM potassium phosphate buffer (pH 7.2), at 4 °C for 16 h to disrupt the organelles. The supernatant obtained by centrifugation at 139,000 x g for 2 h could be regarded as a peroxisomal matrix fraction, judged from the predominant localization of catalase, and pellets as a peroxisomal membrane fraction because about a half of the peroxisomal protein was recovered in this fraction (Table 1). The results suggested that peroxisomal malate synthase was mainly localized in the peroxisomal matrix as was the case of catalase.

Table 1. Intraperoxisomal localization of malate synthase in *Candida tropicalis*

Fraction	Catalase (mmol/min)	Malate synthase (nmol/min)	Protein (mg)
Peroxisomes	36.3	1,870	6.74
Matrix fraction	35.2	1,710	3.31
Membrane fraction	3.1	300	3.37

2. Purification of peroxisomal malate synthase

The particulate fraction obtained from the protoplast homogenate (20,000 x g pellets) was used as the source of peroxisomal malate synthase because preparation of the fraction in a large quantity was easy and the enzyme was detected only in peroxisomes present in this fraction. The 127,000 x g supernatant obtained as described in "MATERIALS AND METHODS" was applied to a column (2.0 x 25 cm) of DEAE-Sephacel equilibrated with 10 mM potassium phosphate buffer (pH 7.2). The column was washed with the same buffer (240 ml) and proteins were eluted with a linear gradient of KCl prepared from 300 ml of 10 mM potassium phosphate buffer (pH 7.2) and 300 ml of the same buffer containing 1 M KCl. The enzyme was not adsorbed on DEAE-Sephacel, while large parts of other proteins were adsorbed on this column and eluted with the KCl gradient (Fig. 1). The enzyme-containing fractions (No. 5 - 8, 32 ml) were pooled and concentrated to 3.5 ml by ultrafiltration with an Amicon Diaflo PM 10 membrane. The enzyme solution was further applied to a column (2.0 x 75 cm) of Sepharose 6B equilibrated with 10 mM potassium phosphate buffer (pH 7.2), and proteins were eluted with the same buffer (Fig. 2). The

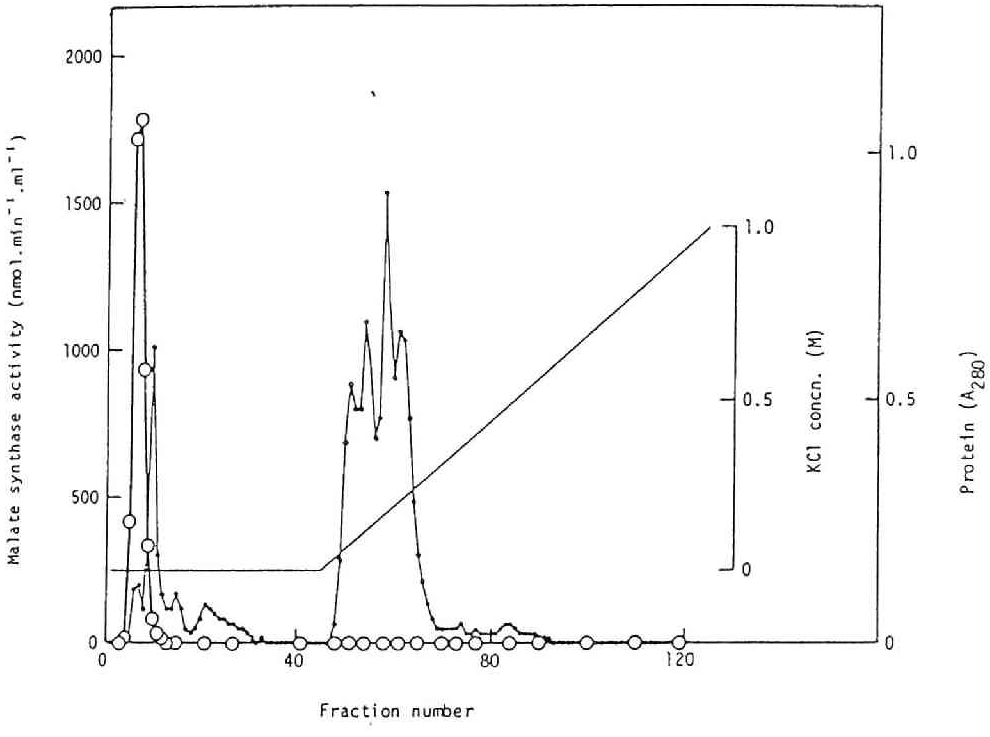


Fig. 1. DEAE-Sephacel column chromatography of malate synthase.

Volume of each fraction, 8.0 ml; flow rate, 27.8 ml/h. (○), Enzyme activity; (●), protein; (—), KCl concentration.

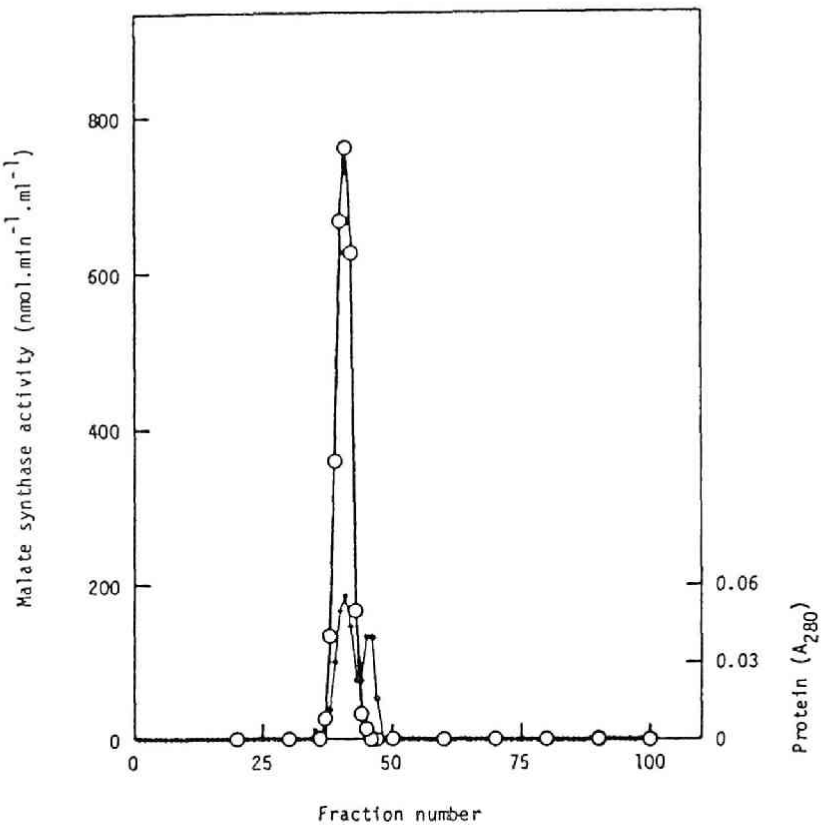


Fig. 2. Sepharose 6B column chromatography of malate synthase.

Volume of each fraction, 4.0 ml; flow rate, 16.3 ml/h. (○), Enzyme activity; (●), protein.

Table 2. Purification of peroxisomal malate synthase from alkane-grown *Candida tropicalis*

Fraction	Total protein (mg)	Total activity (nmol/min)	Yield (%)	Specific activity ($nmol.min^{-1}.mg^{-1}$)	Purification (fold)
Particulate fraction	2,200	174,000	100	79.1	1
127,000 x g supernatant	277	154,000	89	556	7.0
DEAE-Sephacel eluate	7.44	126,000	72	16,900	210
Sepharose 6B eluate	5.41	102,000	59	18,900	240

active fractions (No. 38 - 43, 24 ml) were collected and concentrated to 3.7 ml. This preparation was used as the purified peroxisomal malate synthase. The purity of the enzyme after Sepharose 6B column chromatography was confirmed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, only one protein band being detected on the gel. All purification steps except for the preparation of yeast protoplasts were carried out at 0 - 4 °C.

Table 2 summarizes a typical result on the enzyme purification. The enzyme was purified about 240-fold with a yield of 59 %. The purified enzyme was stable at 0 - 4 °C at least for two weeks, but freeze/thaw inactivated the enzyme significantly.

3. Estimation of molecular mass of the enzyme

Molecular mass of the subunit of malate synthase was determined by sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis. The presence of one kind of subunit with molecular mass of 61 kDa was demonstrated as in Fig. 3. Molecular mass of the native form of the enzyme was estimated to be 250 kDa by gel filtration on a Sepharose 6B column (Fig. 4). By analytical ultracentrifugation, one

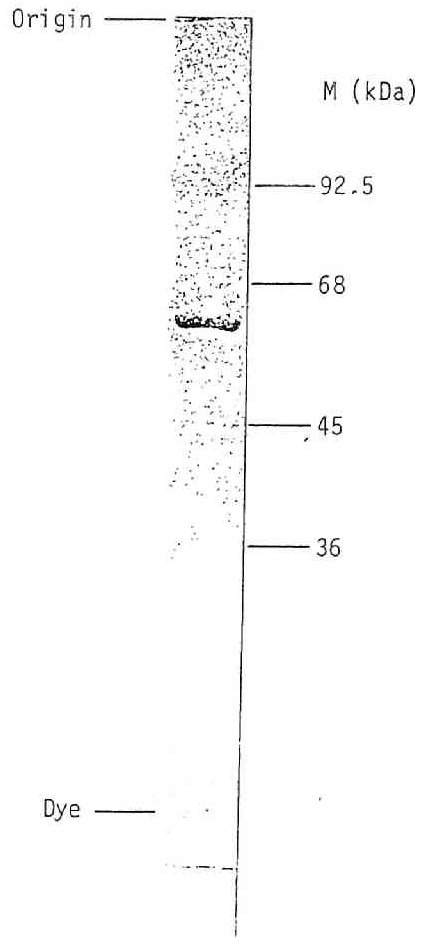


Fig. 3. Sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis of purified peroxisomal malate synthase.

The concentrated Sepharose 6B eluate (2.54 µg protein) was applied on 10 % acrylamide gel. The marker proteins used were phosphorylase b (M, 92.5 kDa), bovine serum albumin (M, 68 kDa), ovalbumin (M, 45 kDa) and lactate dehydrogenase (M, 36 kDa).

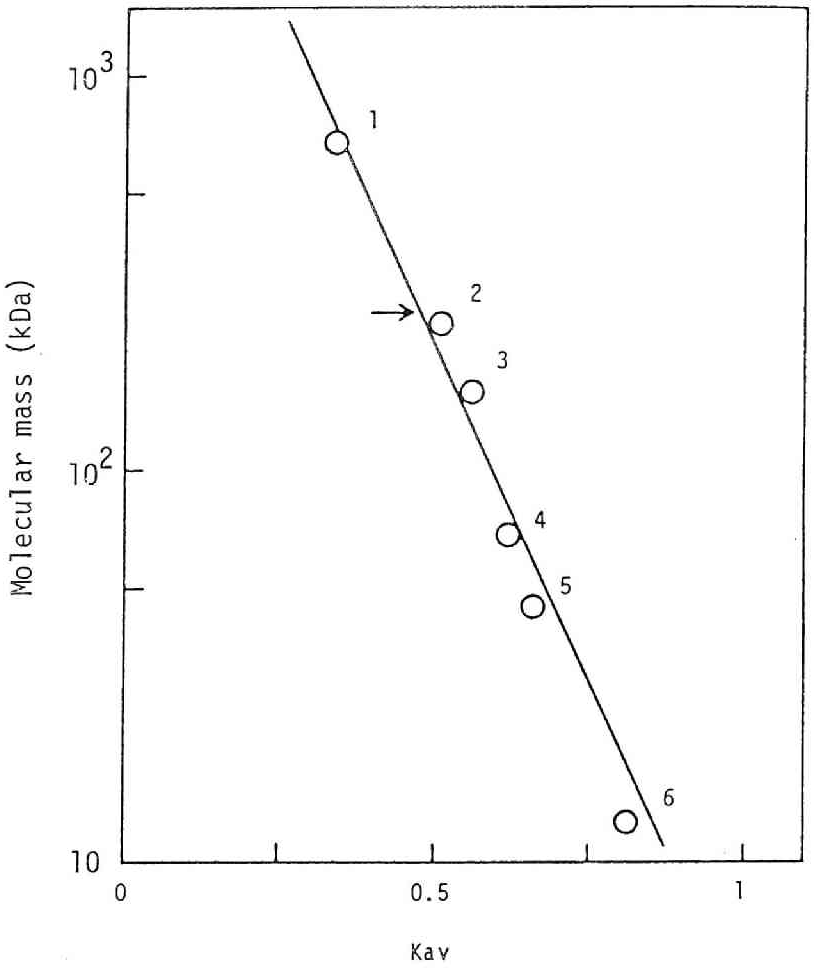


Fig. 4. Estimation of molecular mass of peroxisomal malate synthase by gel chromatography.

The arrow indicates the elution point of malate synthase. Following proteins were used as markers: 1, Thyroglobulin (M, 669 kDa); 2, catalase (M, 232 kDa); 3, aldolase (M, 158 kDa); 4, bovine serum albumin (M, 68 kDa); 5, ovalbumin (M, 45 kDa); and 6, cytochrome c (M, 12.5 kDa).

major peak accompanied by two minor peaks was observed (Fig. 5). The $S_{20,w}$ value of the major peak was calculated to be 12.8 S, the molecular mass of the enzyme being estimated to be 250 kDa by a sedimentation equilibrium method. The minor peaks observed were suggested to be monomeric and aggregated forms of the enzyme. These results indicate that peroxisomal malate synthase from C. tropicalis consists of four identical subunits, that is, the enzyme being a homo-tetramer.

4. Properties of peroxisomal malate synthase

The properties of the enzyme are summarized in Table 3. The enzyme showed the highest activity at pH 8.0. The Km values measured at pH 8.0 were 4.7 mM for Mg^{2+}, 80 μM for acetyl-CoA and 1.0 mM for glyoxylate. Other divalent metal ions, such as Mn^{2+}, Ca^{2+} and Ba^{2+}, could not substitute Mg^{2+}. Glycollate, oxalate, pyruvate, oxalacetate and propionyl-CoA did not serve as substrate.

DISCUSSION

Kollor and Kindl (2) reported that malate synthase of

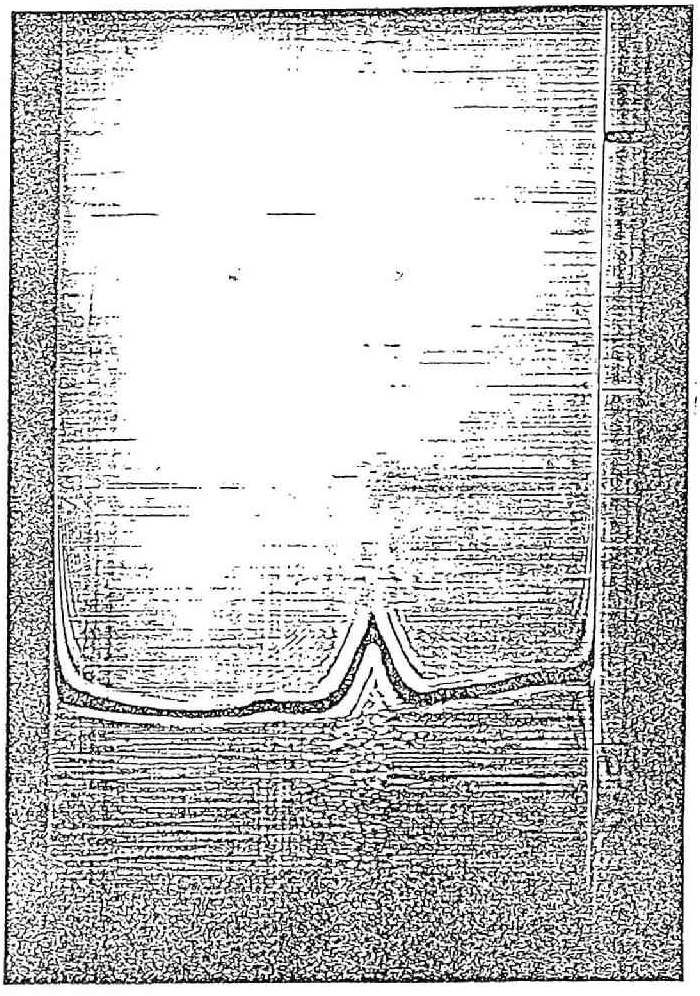


Fig. 5. Sedimentation profile of peroxisomal malate synthase in ultracentrifugation.

The enzyme, dissolved in 50 mM potassium phosphate buffer (pH 7.2) containing 0.3 M KCl at a protein concentration of 1.84 mg/ml, was centrifuged in An-H rotor fitted with a double-sector centerpiece cell at 20 °C and a rotor speed of 59,780 rpm. The photograph of the Schlieren pattern was taken 39 min after this speed was attained.

Table 3. Properties of peroxisomal malate synthase

Molecular mass (kDa)	
Subunit	61
Native enzyme	250
Number of subunit	4
Specific activity ($\mu mol.min^{-1}.mg\ protein^{-1}$)	18.9
Optimum reaction pH	8.0
Substrate specificity	Glyoxylate and acetyl-CoA
K_m value for	
Mg^{2+} (mM)	4.7
Acetyl-CoA (μM)	80
Glyoxylate (mM)	1.0

cucumber cotyledon was associated with the membrane of glyoxysomes. In contrast, malate synthase of yeast peroxisomes was easily solubilized from peroxisomes by transferring them from a hypertonic buffer to a hypotonic buffer to disrupt the peroxisomes. These results suggest that this enzyme was localized in the peroxisomal matrix. Similar localization of malate synthase in microbody matrix has also been proved with ethanol/methylamine-grown Candida utilis by cytochemical staining method (3).

Various molecular mass values have been reported for malate synthase from different sources (Table 4). The bacterial enzymes are monomeric and their molecular masses are relatively small (M, 52 - 60 kDa) (16,17), while the enzyme from Euglena gracilis (M, 350 kDa) (18) and that from baker's yeast (M, 180 kDa) (19) are dimeric and trimeric forms, respectively, with molecular masses of a medium size. On the other hand, the molecular masses of malate synthase from higher plants are considerably larger (M, > 500 kDa) and the enzymes seem to be octamers or multimers consisting of identical subunits (2,20). By gel filtration on Sepharose 6B and by analytical ultracentrifugation, the molecular mass of peroxisomal malate

Table 4. Comparison of molecular mass and subunit number of malate synthase from different sources

Source	Molecular mass of native enzyme (kDa)	Molecular mass of subunit (kDa)	Number of subunit	Reference
Cotyledons of dark-germinated cotton (*Gossypium hirsutum*)	750	—	—	(22)
Cotyledons of cucumber seedling (*Cucumis sativus*)	540	63	8	(2)
Castor bean endosperm (*Ricinus communis*)	575	64	—	(20)
Euglena gracilis	350	175	2	(18)
Baker's yeast	180	60	3	(19)
Peroxisomes of yeast (*Candida tropicalis*)	250	61	4	This work
Thermophilic *Bacillus* sp.	60	60	1	(17)
Escherichia coli				(16)
glucose-grown cells	52	52	1	
acetate-grown cells	54	54	1	

synthase from Candida tropicalis was estimated to be 250 kDa, and the native enzyme was found to be a tetramer consisting of identical subunits of molecular mass of 61 kDa.

Peroxisomal malate synthase from C. tropicalis showed a substrate specificity similar to that of the enzyme purified from other sources (21,22). Only glyoxylate and acetyl-CoA served as substrates for the enzyme. Propionyl-CoA, one of the final degradation products derived from odd-chain alkanes, was not a substrate. Mg^{2+}, which was essential for enzyme activity, could not be replaced by other divalent metal ions.

As reported previously (10), peroxisomal carnitine acetyltransferase and malate synthase utilize acetyl-CoA produced via fatty acid β-oxidation system in peroxisomes of alkane-utilizing C. tropicalis. The former had the optimal pH at 8.0 and the Km value for acetyl-CoA of 42 μM. The affinity for malate synthase of acetyl-CoA (Km = 80 μM) is comparable to that for carnitine acetyltransferase, these results suggesting that the glyoxylate cycle as well as the acetylcarnitine shuttle between peroxisomes and mitochondria might be functional in the yeast assimilating alkanes.

SUMMARY

Malate synthase, one of the key enzymes in the glyoxylate cycle, was purified from peroxisomes of alkane-grown yeast, Candida tropicalis. The enzyme was mainly localized in the matrix of peroxisomes, judging from subcellular fractionation followed by exposure of the organelles to hypotonic conditions. The molecular mass of this peroxisomal malate synthase was determined to be 250 kDa by gel filtration on a Sepharose 6B column as well as by ultracentrifugation. On sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis, the molecular mass of the subunit of the enzyme was demonstrated to be 61 kDa. These results revealed that the native form of this enzyme was homo-tetrameric. Peroxisomal malate synthase showed the optimal activity at pH 8.0 and absolutely required Mg^{2+} for enzymatic activity. The Km values for Mg^{2+}, acetyl-CoA and glyoxylate were 4.7 mM, 80 μM and 1.0 mM, respectively.

REFERENCES

(1) Beevers,H. (1979) Ann. Rev. Plant Physiol. 30, 159.

(2) Koller,W. & Kindl,H. (1977) Arch. Biochem. Biophs. 181, 236.

(3) Zwart,K.B., Veenhuis,M., Plat,G. & Harder,W. (1983) Arch. Microbiol. 136, 28.

(4) Goodman,D.B.P., Davis,W.L. & Jones,R.G. (1980) Proc. Natl. Acad. Sci. USA 77, 1521.

(5) Jones,C.T. (1980) Biochem. Biophys. Res. Commun. 95, 849.

(6) Osumi,M., Miwa,N., Teranishi,Y., Tanaka,A. & Fukui,S. (1974) Arch. Microbiol. 99, 181.

(7) Osumi,M., Fukuzumi,F., Teranishi,Y., Tanaka,A. & Fukui,S. (1975) Arch. Microbiol. 103, 1.

(8) Fukui,S. & Tanaka,A. (1979) Trends Biochem. Sci. 4, 246.

(9) Tanaka,A., Osumi,M. & Fukui,S. (1982) Ann. N. Y. Acad. Sci. 386, 183.

(10) Ueda,M., Tanaka,A. & Fukui,S. (1982) Eur. J. Biochem. 124, 205.

(11) Ueda,M., Tanaka,A. & Fukui,S. (1984) Eur. J. Biochem.

138, 445.

(12) Uchida,M., Ueda,M., Matsuki,T., Okada,H., Tanaka,A. & Fukui,S. (1985) Agric. Biol. Chem. 50, 127.

(13) Dixon,G.H. & Kornberg,H.L. (1959) Biochem. J. 72, 3.

(14) Nabeshima,S., Tanaka,A. & Fukui,S. (1977) Agric. Biol. Chem. 41, 275.

(15) Lowry,O.H., Rosebrough,N.J., Farr,A.L. & Randall,R.J. (1951) J. Biol. Chem. 193, 265.

(16) Falmagne,P. & Wiame,J.M. (1973) Eur. J. Biochem. 37, 415.

(17) Sundaram,T.K., Chell,R.M. & Wilkinson,A.E. (1980) Arch. Biochem. Biophys. 199, 515.

(18) Woodcock,E. & Merrett,M.J. (1978) Biochem. J. 173, 95.

(19) Durchschlag,H., Biedermann,G. & Eggerer,H. (1981) Eur. J. Biochem. 114, 255.

(20) Bowden,L. & Lord,J.M. (1978) Plant Physiol. 61, 259.

(21) Dixon,G.H., Kornberg,H.L. & Lund,P. (1960) Biochim. Biophys. Acta 41, 217.

(22) Miernyk,J.A. & Trelease,R.N. (1981) Phytochem. 20, 2657.

Chapter 3. Comparison and subcellular distribution of isocitrate lyase and malate synthase in *Candida tropicalis* cells grown on different carbon sources

INTRODUCTION

Candida tropicalis cells grown on *n*-alkanes as the sole source of carbon and energy have profuse numbers of peroxisomes. These organelles contain catalase and the key enzymes of the glyoxylate cycle (isocitrste lyase and malate synthase) together with the enzymes involved in alkane metabolism. During the growth of the yeast on an *n*-alkane mixture (C_{10}- C_{13}), the glyoxylate cycle is supposed to function between peroxisomes and mitochondria which contain other glyoxylate cycle enzymes common to the tricarboxylic acid cycle (1).

This yeast can utilize glucose, acetate and propionate as carbon sources (2). The cells grown on glucose or acetate have a few peroxisomes, that is, pre-existing peroxisomes. In contrast to alkane-grown cells containing a large number of small peroxisomes, the cells grown on

propionate have been observed to have a small number of large peroxisomes. In this case, catalase, which is localized in peroxisomes of alkane-grown cells and is a so-called marker enzyme of peroxisomes, is mostly found in cytosol. The organelles of propionate-grown cells may be regarded as immature peroxisomes.

As described in Chapter 1 and 2 in Part II, isocitrate lyase (3) and malate synthase (4) have been purified from peroxisomes of n-alkane-grown cells and their properties were clarified, because these enzymes as well as catalase have indispensable roles in peroxisomes of the yeast cells. Development of peroxisomes is connected with the transfer of enzymes to organelles. Therefore, it will be important to compare the properties of these enzymes purified from the yeast cells having different developing states of peroxisomes.

This paper deals with the subcellular localization and some properties of isocitrate lyase and malate synthase in C. tropicalis grown on different carbon sources.

MATERIALS AND METHODS

1. Cultivation of yeast

Candida tropicalis pK 233 (ATCC 20336) was cultivated aerobically at 30 °C in a medium of initial pH 6.0, as in Chapter 1 of Part I. Glucose 16.5 g/l, sodium acetate 13.6 g/l, sodium propionate 10 g/l, or n-alkane mixture (C_{10} - C_{13}) 10 ml/l was used as carbon and energy source.

2. Preparation of enzyme sources

Cells harvested at exponential growth phase were washed twice with deionized water and once with 50 mM potassium phosphate buffer (pH 7.2). Washed cells were suspended in the same buffer and homogenized with a Braun cell homogenizer (for 150 s per 1 g dry cell). The homogenized cell suspension was centrifuged at 127,000 x g for 1 h. The supernatant obtained was used as cell-free extract.

3. Immunochemical procedures

Rabbit antisera against purified peroxisomal isocitrate lyase (Ps-ICL) and malate synthase (Ps-MS) were prepared as in Chapter 1 of Part II (5). Immunochemical titration and

analysis were performed as in Chapter 1 of Part II.

4. Subcellular fractionation

Yeast protoplasts were prepared as in Chapter 1 of Part I (6). Protoplasts were homogenized with a teflon homogenizer and fractionated by differential centrifugation. Fractions obtained were as follows: P_2 fraction (20,000 x g pellets), mitochondria and peroxisomes; P_3 fraction (139,000 x g pellets), microsomes; and S_3 fraction (139,000 x g supernatant), cytosol.

5. Purification of isocitrate lyases and malate synthases

Purification of peroxisomal isocitrate lyase (Ps-ICL) and malate synthase (Ps-MS) from n-alkane-grown cells were reported in preceding chapters (3,4). Isocitrate lyase and malate synthase from acetate-grown (A-ICL, A-MS) and propionate-grown (P-ICL, P-MS) whole cells were purified from the respective cell-free extracts by means of DEAE-Sephacel column chromatography followed by Sephacryl S-300 column chromatography (isocitrate lyase) or by Sepharose 6B column chromatography (malate synthase) under the same conditions as in Chapters 1 and 2 of Part II (3,4).

Purification of isocitrate lyase from glucose-grown cells (G-ICL) was performed as in Chapter 1 of Part II (3). Malate synthase from glucose-grown cells (G-MS) was purified by means of DEAE-Sephacel column chromatography followed by CM-Sepharose CL-6B and Sepharose 6B column chromatographies. Purification of the enzymes was carried out at 0 - 4 °C.

6. Enzyme assay and protein measurement

Isocitrate lyase was assayed by following the increase in absorbance of glyoxylate-phenylhydrazone at 324 nm and, malate synthase activity was measured by following the decrease in absorbance of acetyl-CoA at 232 nm as in Chapter 1 of Part II. Protein was quantified according to Lowry et al. (7).

7. Limited proteolysis of isocitrate lyase and malate synthase subunits

After each isocitrate lyase and malate synthase had been dissociated into subunits on 10 % polyacrylamide slab-gels in the presence of sodium dodecylsulfate, the gel areas corresponding to the subunits were excised and electrophoresed with proteases as described by Cleveland et al.

(8), except that 12.5 % acrylamide gels were used as the digesting gels. Peptide fragments were detected by the silver staining method.

8. Analysis of amino terminus

Amino-terminal analysis was performed by the dansylation method of Gray (9).

RESULTS

1. Activities of isocitrate lyase and malate synthase in cells grown on different carbon sources

The levels of isocitrate lyase and malate synthase were compared with the cell-free extracts from the cells harvested at the mid-exponential growth phase on different carbon sources (Table 1). The levels of isocitrate lyase activity in propionate-, n-alkane- and acetate-grown cells were about three to seven times higher than that in glucose-grown cells. As for malate synthase activity, propionate-grown cells had much higher level than glucose-grown cells. The level of malate synthase activity was almost the same between acetate-grown and n-alkane-grown cells, about three times higher than that in glucose-grown cells.

Table 1. Levels of isocitrate lyase, malate synthase and catalase activities in Candida tropicalis cells grown on different carbon sources

Cells	Isocitrate lyase ($nmol.min^{-1}.mg\ protein^{-1}$)	Malate synthase ($nmol.min^{-1}.mg\ protein^{-1}$)	Catalase ($\mu mol.min^{-1}.mg\ protein^{-1}$)
Glucose-grown	171	23	736
Acetate-grown	1,180	75	793
Propionate-grown	613	400	3,750
n-Alkane-grown	529	65	8,010

2. Immunochemical properties

The immunochemical properties of isocitrate lyase were examined by using an antiserum against isocitrate lyase from peroxisomes of n-alkane-grown cells (Ps-ICL). The immunochemical titration revealed that the respective enzymes were completely precipitated by the anti-(Ps-ICL) antiserum (Fig. 1A). Moreover, the immunochemical analysis showed that the equivalent points of the respective enzymes were the same (Fig. 2A), indicating that inactive protein was absent in these cells. These results indicated that isocitrate lyase from the cells grown on different carbon sources was immunochemically indistinguishable and that only one kind of the active enzyme was present in the cells regardless of the growth substrates. Same results were also obtained on malate synthase with an antiserum against malate synthase from peroxisomes of n-alkane-grown cells (Ps-MS) (Figs. 1B and 2B).

3. Subcellular localization of isocitrate lyase and malate synthase

As shown in Table 2, isocitrate lyase and malate

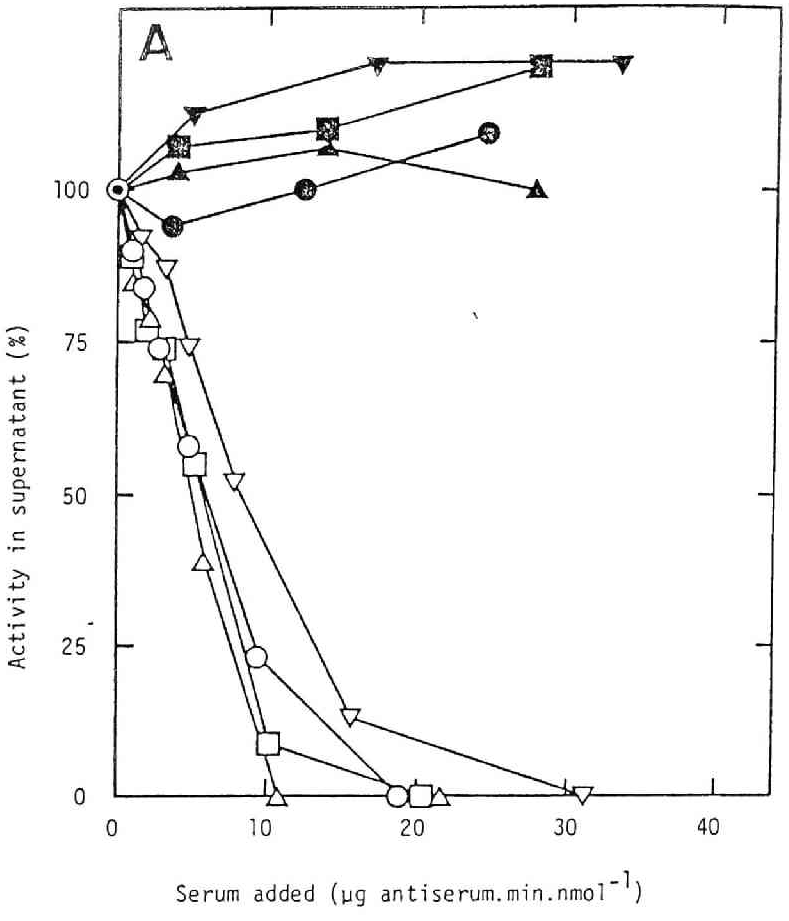
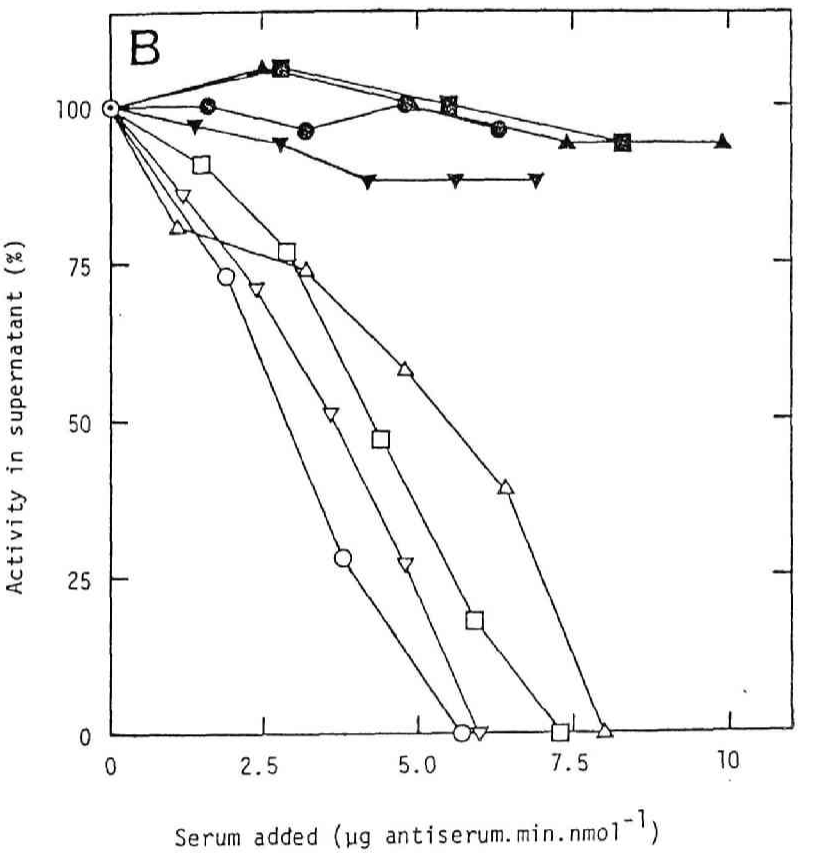


Fig. 1. Immunochemical titration of isocitrate lyases with anti-(Ps-ICL) antiserum (A) and malate synthases with anti-(Ps-MS) antiserum (B).

Fixed amounts of cell-free extracts from glucose-grown (ICL, 339; MS, 467 nmol/min), acetate-grown (ICL, 295; MS, 300 nmol/min), propionate-grown (ICL, 205; MS, 533 nmol/min), and n-alkane-grown cells (ICL, 312; MS, 267 nmol/min) were titrated with the respective antisera. (○,●), Glucose-grown; (△,▲), acetate-grown; (▽,▼), propionate-grown; and (□,■), n-alkane-grown cells. (○,△,▽,□), Anti-(Ps-ICL) antiserum (A) or anti-(Ps-MS) antiserum; (●,▲,▼,■), control serum (A and B).


B
100
75
50
25
0
0
2.5
5.0
7.5
10
Activity in supernatant (%)
Serum added (µg antiserum.min.nmol^{-1})

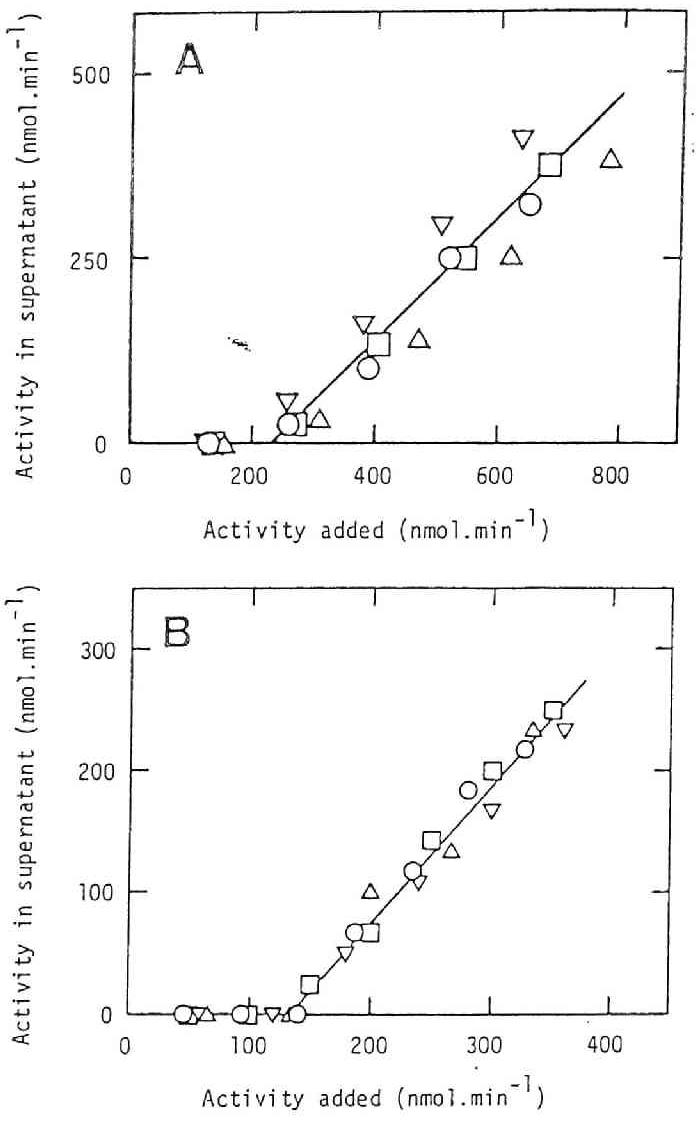


Fig. 2. Immunochemical analysis of isocitrate lyases with anti-(Ps-ICL) antiserum (A) and malate synthases with anti-(Ps-MS) antiserum (B).

(A), A fixed amount of anti-(Ps-ICL) antiserum (3,190 µg) was reacted with different amounts of cell-free extracts from glucose-grown (○), acetate-grown (△), propionate-grown (▽), and n-alkane-grown cells (□). (B), A fixed amountof anti-(Ps-MS) antiserum (740 µg) was reacted with the cell-free extracts in a similar manner as in (A).

synthase, which were peroxisome-associated enzymes in n-alkane-grown cells, were predominantly localized in S_3 fraction (cytosol) of propionate-grown cells. In glucose-grown cells, large parts of the enzymes were present in P_2 fraction (mitochondria + peroxisomes) as in n-alkane-grown cells. Similar results were also obtained on the localization of catalase, the marker enzyme of peroxisomes. In acetate-grown cells, isocitrate lyase and malate synthase were present in P_2 fraction as well as in S_3 fraction. In general, higher ratios of malate synthase were detected in S_3 fractions than those of isocitrate lyase and catalase. In all cases, the enzymes showed more or less a diverse distribution among the subcellular fractions in spite of the fact that these cells contained only one kind of the respective enzymes as mentioned above.

4. Properties of isocitrate lyase and malate synthase purified from cells grown on different carbon sources

The active forms of respective isocitrate lyase were eluted in a single peak on gel filtration on Sepharose 6B and their molecular masses were all estimated to be approximately 130 kDa. On polyacrylamide slab-gel electro-

Table 2. Subcellular distribution of isocitrate lyase, malate synthase and catalase in Candida tropicalis cells grown on different carbon sources

Cells	Relative activity (%)								
	Isocitrate lyase			Malate synthase			Catalase		
	P_2	P_3	S_3	P_2	P_3	S_3	P_2	P_3	S_3
Glucose-grown	75	5	20	56	11	33	87	1	12
Acetate-grown	60	5	35	47	4	49	67	7	26
Propionate-grown	18	7	75	27	14	59	37	9	54
n-Alkane-grown	76	2	22	68	6	27	86	2	12

P_2, 20,000 x g pellets (mitochondria + peroxisomes); P_3, 139,000 x g pellets (microsomes); S_3, 139,000 x g supernatant (cytosol).

phoresis in the presence of sodium dodecylsulfate, isocitrate lyase gave single peptide bands whose molecular masses were calculated to be 65 kDa, the results indicating that isocitrate lyase was homo-dimeric. Molecular masses of the active forms of respective malate synthase were determined to be 250 kDa, and those of the subunits of the enzymes to be 61 kDa, that is, malate synthase was homo-tetrameric.

Specific activities of purified isocitrate lyase and malate synthase were almost the same, respectively, independent of the enzyme sources (Table 3). Km values for Mg^{2+} and isocitrate were almost the same among respective isocitrate lyase. In the case of malate synthase, Km values for Mg^{2+} were almost the same among the enzymes, but those for acetyl-CoA and glyoxylate were relatively different.

Amino-terminal analysis by dansylation showed that alanine occupied amino-terminal positions of each isocitrate lyase and glycine occupied that of each malate synthase (Table 3).

To compare peptide components of isocitrate lyase and malate synthase from the cells grown on different carbon sources, their digested peptide fragments were prepared by

Table 3. Properties of isocitrate lyases and malate synthases purified from Candida tropicalis cells grown on different carbon sources

(A) Isocitrate lyase

Property	Ps-	G-	A-	P-
Molecular mass (kDa)				
Active form	130	130	130	130
Subunit	65	65	65	65
Km (mM)				
Mg^{2+}	0.13	0.14	0.24	0.24
Isocitrate	1.7	1.4	2.9	2.9
Specific activity ($nmol.min^{-1}.mg\ protein^{-1}$)	10.9	8.75	8.99	8.10
N-terminal amino acid	Ala	Ala	Ala	Ala

(B) Malate synthase

Property	Ps-	G-	A-	P-
Molecular mass (kDa)				
Active form	250	250	250	250
Subunit	61	61	61	61
Km				
Mg^{2+} (mM)	4.7	3.8	2.8	7.1
Acetyl-CoA (µM)	80	5.6	9.1	56
Glyoxylate	1.0	0.24	0.15	0.10
Specific activity ($nmol.min^{-1}.mg\ protein^{-1}$)	18.9	18.9	15.7	20.3
N-terminal amino acid	Gly	Gly	Gly or Ala	Gly

limited proteolysis with Staphylococcus aureus V8 protease or papain, respectively. Similar peptide maps for the respective enzymes were obtained as shown in Fig. 3.

DISCUSSION

The key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase), localized in peroxisomes of n-alkane-grown yeast Candida tropicalis, have an essential role on assimilation of n-alkanes. These enzymes were also induced in acetate-grown cells, as the case of ethanol-grown cells of Candida utilis and Hansenula polymorpha (10), and in propionate-grown cells, but appreciable parts of these enzymes were localized in cytosol, different from their predominant localization in peroxisomes of n-alkane-grown cells.

There is a possibility that two types of the enzymes, that is, cytosolic and peroxisomal enzymes or precursor-type active or inactive and mature enzymes, are present in the yeast cells since peroxisomal and mitochondrial types of carnitine acetyltransferases were confirmed in this yeast cells grown on n-alkanes (5,11). However, all the active

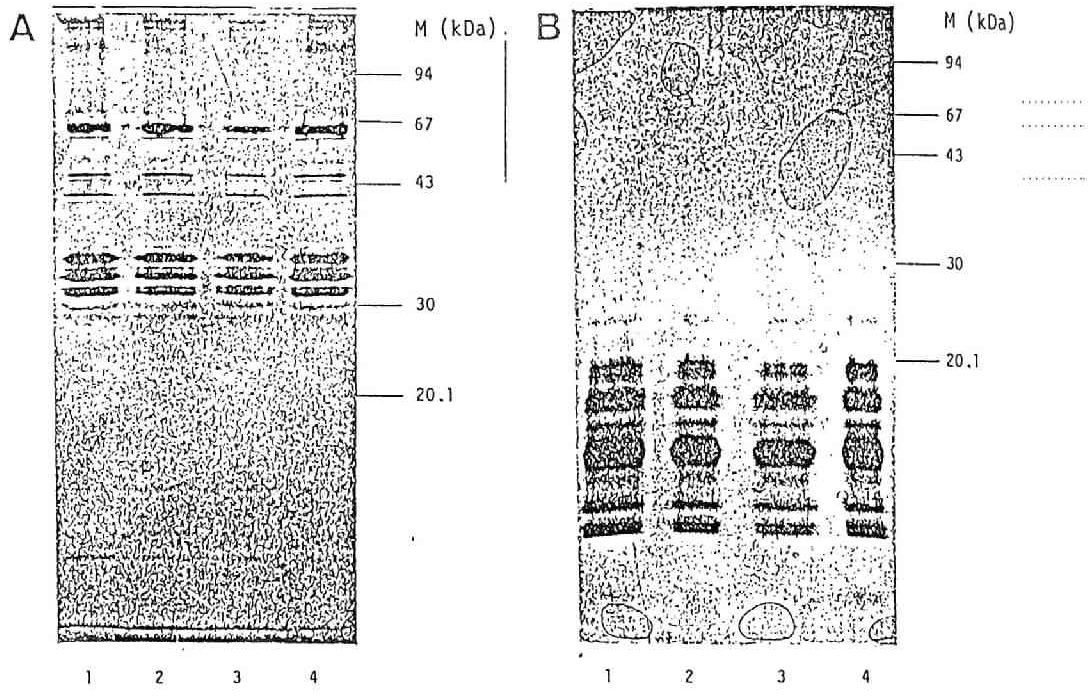
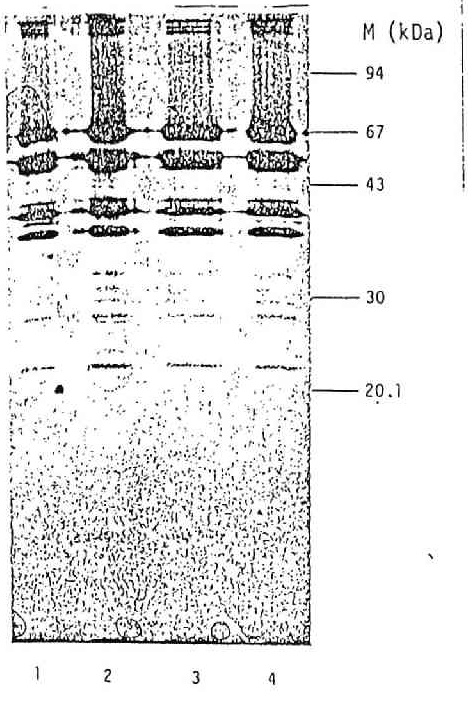
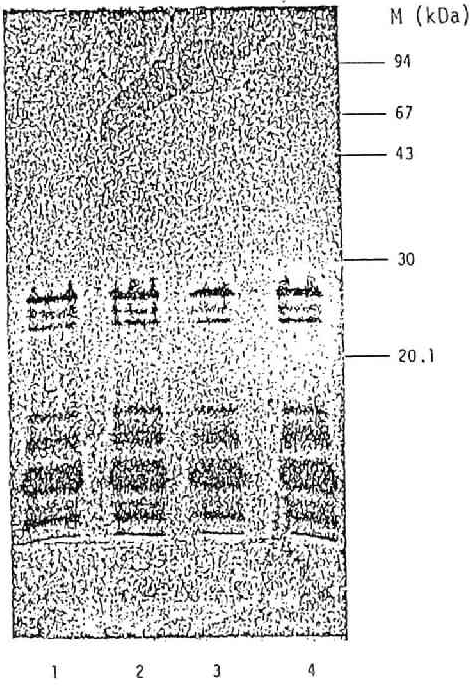


Fig. 3. Limited proteolysis of isocitrate lyases (A and B) and malate synthases (C and D) with Staphylococcus aureus V8 protease (A and C) or papain (Papaya latex protease) (B and D) on sodium dodecylsulfate/polyacrylamide slab-gel (12.5 %) electrophoresis.

Peptides were stained with silver staining reagents. (A and C), Peptide maps of the respective enzymes (10 µg) with S. aureus V8 protease (50 ng); (B and D), peptide maps of the respective enzymes (10 µg) with papain (12 ng). (A and B) 1, G-ICL; 2, A-ICL; 3, P-ICL; 4, Ps-ICL; (C and D) 1, G-MS; 2, A-MS; 3, P-MS; and 4, Ps-MS. The marker proteins used were phosphorylase b (M, 94 kDa), bovine serum albumin (M, 67 kDa), ovalbumin (M, 43 kDa), carbonic anhydrase (M, 30 kDa), and soybean trypsin inhibitor (M, 20.1 kDa).



D



isocitrate lyase and malate synthase purified from the yeast cells grown on different carbon sources showed similar immunochemical and proteinaceous properties ----- molecular masses, amino-terminal amino acids etc. -----, the results suggesting the presence of only one type of the respective enzymes. The immunochenical analysis also indicated the absence of inactive enzyme proteins.

Biosynthesis of isocitrate lyase and malate synthase, which are localized in glyoxysomes, have been studied with Neurospora crassa (12) and cucumber cotyledons (13-16). These results showed that their in vitro translation products had a same size with mature enzymes from glyoxysomes, indicating they had no precursors with leading sequence at N-terminus. Kruse and Kindl (17) reported that cytoplasmic malate synthase from cucumber cotyledons had active monomeric and aggregated forms, different from the glyoxysomal enzyme. However, such different forms of the active emzyme were not detected in the yeast cells.

Propionate-grown C. tropicalis cells seem to have immature peroxisomes, because isocitrate lyase and malate synthase, which are inducibly biosynthesized, are not accomodated in peroxisomes, in contrast to n-alkane-grown

cells containing these enzymes specifically localized in mature peroxisomes. Different subcellular localization of the enzymes might reflect the developing states of the organelles, that is, transportation of the enzymes from cytosol to peroxisomes would be connected with the development of the organelles. The incorporation of these enzymes to peroxisomes might be regulated by the amount of a certain receptor(s) on peroxisomal membranes, which would be deficient on the surface of the organelles in propionate-grown cells. In the case of acetate-grown cells, there is a possibility that isocitrate lyase and malate synthase might be saturated in pre-existing peroxisomes, since the number of peroxisomes in the cells were only a few in spite of the fact that the enzymes were synthesized inducibly.

SUMMARY

The activities of isocitrate lyase and malate synthase ----- the key enzymes of the glyoxylate cycle ----- were fairly high in n-alkane-, acetate-, and propionate-grown cells of Candida tropicalis compared with those in glucose-grown cells. Both enzymes were localized predomi-

nantly in mature peroxisomes of alkane-grown cells and in pre-existing peroxisomes of glucose-grown cells, while the cytosolic enzymes were predominant in propionate-grown cells. In acetate-grown cells, these enzymes were localized in pre-existing peroxisomes as well as in cytosol. Isocitrate lyase and malate synthase were purified from a peroxisome-containing particulate fraction of alkane-grown cells and from whole cells grown on glucose, acetate, and propionate. The respective enzymes showed no significant differences in immunochemical properties, specific activities, molecular masses of active forms amd subunits, and patterns of limited proteolysis with proteases, although malate synthase of alkane- and propionate-grown cells had higher Km values for acetyl-CoA than the enzymes of glucose- and acetate-grown cells. The results indicated that the different forms of the active and inactive enzymes were not present in the yeast cells.

REFERENCES

(1) Tanaka,A., Osumi,M. & Fukui,S. (1982) Ann. N. Y. Acad. Sci. 386, 183.

(2) Ueda,M., Okada,H., Tanaka,A., Osumi,M. & Fukui,S. (1983) Arch. Microbiol. 136, 169.

(3) Uchida,M., Ueda,M., Matsuki,T., Okada,H., Tanaka,A. & Fukui,S. (1986) Agric. Biol. Chem. 50, 127.

(4) Okada,H., Ueda,M. & Tanaka,A. (1986) Arch. Microbiol. 144, 137.

(5) Ueda,M., Tanaka,A. & Fukui,S. (1984) Eur. J. Biochem. 138, 445.

(6) Kawamoto,S., Nozaki,C., Tanaka,A. & Fukui,S. (1978) Eur. J. Biochem. 83, 609.

(7) Lowry,O.H., Rosebrough,N.J., Farr,A.L. & Randall,R.J. (1951) J. Biol. Chem. 193, 265.

(8) Cleveland,D.W., Fischer,S.G., Kirschner,M.W. & Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102.

(9) Gray,W.R. (1972) Methods Enzymol. 25, 121.

(10) Zwart,K.B., Veenhuis,M., Plat,G. & Harder,W. (1983) Arch. Microbiol. 136, 28.

(11) Ueda,M., Tanaka,A. & Fukui,S. (1982) Eur. J. Biochem. 124, 205.

(12) Zimmermann,R. & Neupert,W. (1980) Eur. J. Biochem. 112, 225.

(13) Becker,W.M., Rietzman,H., Weir,E.M., Titus,D.E. &

Leaver,C. (1982) Ann. N.Y. Acad. Sci. 386, 329.

(14) Kindl,H. (1982) Ann. N. Y. Acad. Sci. 386, 314.

(15) Roberts,L.M. & Lord,J.M. (1981) Eur. J. Biochem. 119, 43.

(16) Weir,E.M., Riezman,H., Grienenberger,J.M., Becker,W.M. & Leaver,C.J. (1980) Eur. J. Biochem. 112, 469.

(17) Kruse,C. & Kindl,H. (1983) Arch. Biochem. Biophys. 223, 618.

Part III. Purification of a novel propionate-activating (propionylhydroxamate-forming) enzyme from propionate-grown cells of *Candida tropicalis*

INTRODUCTION

Candida tropicalis grown on *n*-alkanes or higher fatty acids has conspicuous numbers of peroxisomes (microbodies) which play an indispensable role in the fatty acid metabolism (1 - 3). As acetyl-CoA and propionyl-CoA are the final products of yeast peroxisomal β-oxidation of fatty acids with even and odd carbon chains, it will be interesting to investigate the roles of peroxisomes in the metabolism of C_2- and C_3-compounds together with the development of the organelles. In the propionate-grown *C. tropicalis* cells, the size and number of peroxisomes were different from those in the alkane-grown cells. At the same time, it has been found that the presence of a novel propionate-activating enzyme in the propionate-grown cells. These results suggest that this yeast is very useful to study the development of peroxisomes together with the metabolism of propionate in eukaryotic cells.

Part III deals with the propionate-activating enzyme, which is quite different from acetyl-CoA synthetase, in C. tropicalis. Purification and some properties of this novel enzyme are described.

MATERIALS AND METHODS

1. Cultivation of yeast

Candida tropicalis pK 233 (ATCC 20336) was cultivated aerobically at 30 °C in a medium containing sodium acetate (13.6 g/l) or sodium propionate (10.0 g/l) as a sole carbon source, as in Chapter 1 of Part I.

2. Subcellular fractionation

Yeast protoplasts were prepared as in Chapter 1 of Part I in the presence of 0.1 M 2-mercaptoethanol. Protoplasts were homogenized with a teflon homogenizer and fractionated by differential centrifugation. Fractions obtained were as follows: P_2 fraction (20,000 x g pellets), mitochondria and peroxisomes; P_3 fraction (139,000 x g pellets), microsomes; and S_3 fraction (139,000 x g supernatant), cytoplasm.

3. Preparation of enzyme sources

The yeast was cultivated for 24 h at 30 °C. Cells harvested by centrifugation (25 g wet cells) were suspended in 30 ml of 100 mM potassium phosphate buffer (pH 6.5) and homogenized using a Braun homogenizer (for 150 s per 10 g wet cells). The cell homogenate was centrifuged at 126,000 x g for 1 h. The precipitate was discarded and the supernatant was used as the cell-free extract.

4. Enzyme assay

The acetate- and propionate-activating enzymes were assayed as follows. A reaction mixture composed of 100 mM potassium phosphate buffer (pH 7.5), 10 mM $MgCl_2$, 10 mM ATP, 10 mM glutathione, 0.1 mM CoASH, 10 mM potassium acetate or potassium propionate, 200 mM hydroxylamine and an enzyme solution (final volume, 1.0 ml) was incubated at 30 C for 20 min. The reaction was terminated with addition of 0.5 ml of 0.66 M HCl solution containing 10 % trichloroacetic acid and 15 % $FeCl_3.6H_2O$, and the mixture was further incubated at 30 °C for 20 min for the development of color. After centrifugation at 10,000 x g for 10 min,

the absorbance of the resulting supernatant was measured at 540 nm. Runs without CoASH were used as control. In this system, A_{540} values of each 1 μmol of hydroxamic acids from acetyl-CoA and propionyl-CoA were 0.325 and 0.267, respectively.

5. Gel electrophoresis

Polyacrylamide gel electrophoresis in the absence or presence of sodium dodecylsulfate was performed according to the method described by Ueda et al. (4,5).

6. Preparation of acetyl- and propionylhydroxamates

Authentic acetyl- and propionylhydroxamates were prepared by the reaction with hydroxylamine (hydrochloric acid salt) and anhydrous acetic acid and propionic acid at pH 10 and room temperature.

7. Chemicals

DEAE-Sephacel, Sepharose 6B, Sephacyl S-300 and Blue-Sepharose CL-6B were obtained from Pharmacia (Uppsala, Sweden); ATP from P-L Biochemicals Inc. (Milwaukee, WI, USA); CoA, acetyl-CoA and propionyl-CoA from Sigma (St.

Louis, MO, USA); glutathione reduced form from Wako Pure Chemicals (Osaka, Japan). Other chemicals were also purchased from commercial sources.

RESULTS

1. Induction and subcellular localization of propionate-activating enzyme

Crude enzyme solutions were prepared from acetate-grown cells and propionate-grown cells harvested at the respective exponential growth phase, and applied to DEAE-Sephacel columns (2.0 x 27 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2). Each column was washed with the buffer and then short-chain fatty acid-activating enzymes were eluted with a linear concentration gradient of KCl prepared from 200 ml of the buffer containing 0.05 M KCl and the same volume of the buffer containing 0.25 M KCl. As shown in Fig. 1A, acetate-grown cells contained one enzyme fraction (I) which could activate acetate and propionate. On the other hand, two types of the enzymes (II and III) were detected in propionate-grown cells. One (II) activated both acetate and propionate. On the contrary, the other (III)

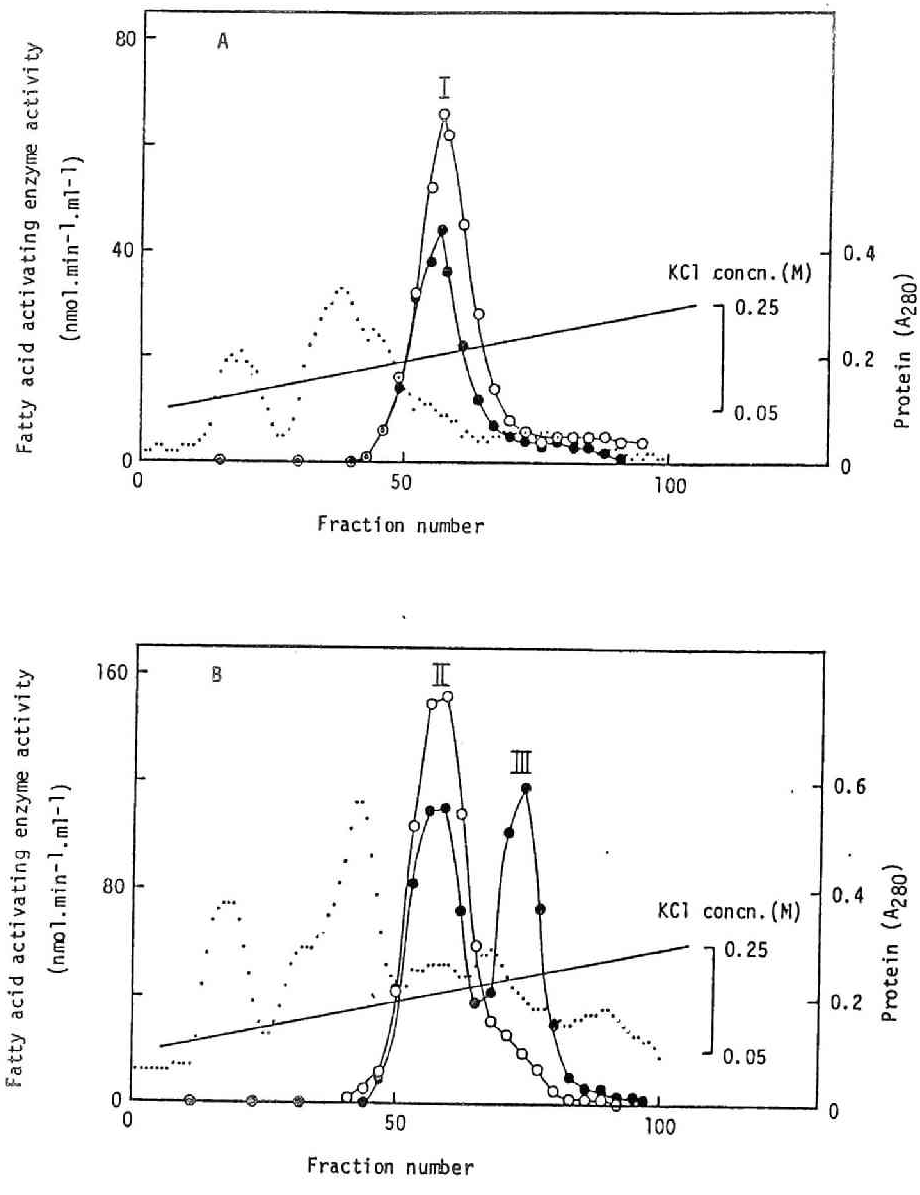


Fig. 1. DEAE-Sephacel column chromatography of short-chain fatty acid-activating enzymes from acetate-grown cells (A) and propionate-grown cells (B) of Candida tropicalis.

(○), Enzyme activity for acetate; (●), enzyme activity for propionate; (—), KCl concentration; (····), protein

showed a high activity toward propionate but a low activity toward acetate (Fig. 1B). This type of the activating enzyme was not observed in acetate-grown cells. Thus, the propionate-specific enzyme (III) was found to be induced in propionate-utilizing cells. The enzyme I from acetate-grown cells and II from propionate-grown cells showed a similar substrate specificity for short-chain fatty acids, activating both acetate and propionate (Fig. 2). Hence, these enzymes could be regarded as an acetate-activating enzyme. On the other hand, the substrate specificity of the enzyme III was quite different from that of the acetate-activating enzyme, being not so active for acetate but exhibiting the maximum activity for propionate. Therefore, the enzyme III was named as the propionate-activating enzyme.

The acetate-activating enzyme was exclusively localized in the cytoplasmic fraction (S_3) of acetate-grown cells (Fig. 3A) and of propionate-grown cells (data not shown). Similarly, the propionate-activating enzyme was also found in S_3 (Fig. 3B).

2. Purification of the propionate-activating enzyme

The cell-free extract was applied to a DEAE-Sephacel

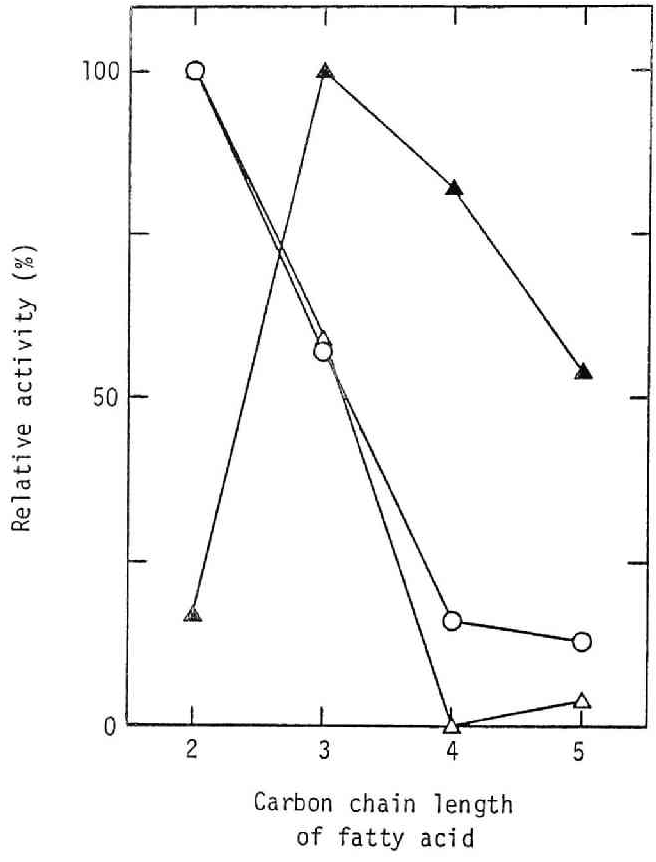


Fig. 2. Substrate specificity of short-chain fatty acid-activating enzymes obtained from acetate-grown cells and propionate-grown cells of Candida tropicalis.

Enzyme I from acetate-grown cells (○), enzyme II (△) and enzyme III (▲) from propionate-grown cells were prepared as shown in Fig. 1. Enzyme activity for acetate (enzymes I and II) or for propionate (enzyme III) was expressed as 100 %.

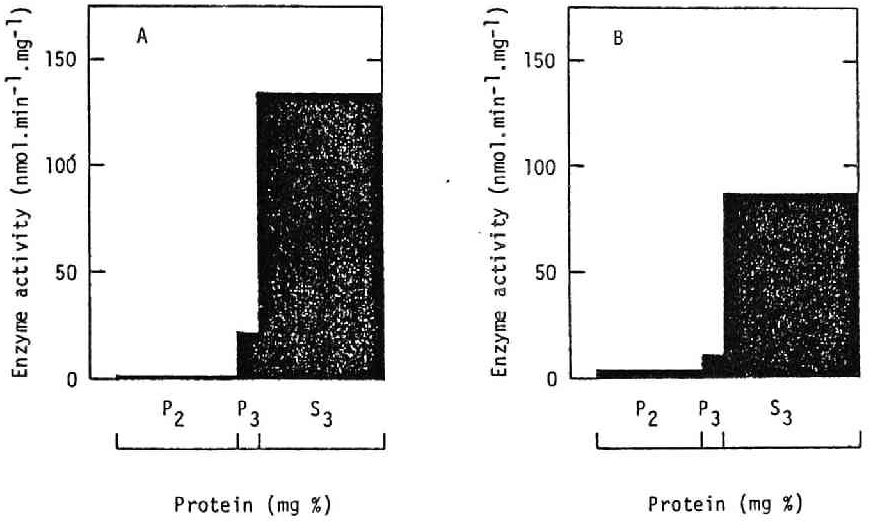


Fig. 3 Subcellular localization of short-chain fatty acid-activating enzymes in Candida tropicalis

A, Enzyme activity in acetate-grown cells measured with acetate as substrate: B, Enzyme activity in propionate-grown cells measured with propionate as substrate. P_2, 20,000 x g pellets (peroxisomes + mitochondria); P_3, 139,000 x g pellets (microsomes); S_3, 139,000 x g supernatant (cytosol).

column (2.0 x 27 cm) equilibrated with 10 mM potassium phosphate buffer (pH 6.5) and proteins were eluted with a linear concentration gradient of KCl prepared from 300 ml of 10 mM potassium phosphate buffer (pH 6.5) containing 0.10 M KCl and the same volume of the buffer containing 0.25 M KCl. The propionate-activating activities were obtained from the column in two peaks as described above. The enzyme eluted below 0.15 M KCl was the acetate-activating enzyme , while the enzyme eluted above 0.15 M KCl was regarded as a novel propionate-activating enzyme. Fractions containing the latter (No. 39 - 50, 72 ml) were pooled, concentrated to 6.8 ml with a Diaflo membrane filter, and applied to a Sepharose 6B column (2.0 x 74 cm) equilibrated with 10 mM potassium phosphate buffer (pH 6.5). The enzyme fractions (No. 46 - 52, 21 ml) were concentrated to 5.8 ml, and applied to a Blue-Sepharose CL-6B column (2.0 x 20 cm) equilibrated previously with the same buffer. The enzyme was eluted in a single peak without adsorption on the column. The fractions exhibiting the enzyme activity (No. 4 - 7, 20 ml) were used as a purified propionate-activating enzyme preparation. The enzyme fractions eluted below 0.15 M KCl on the DEAE-Sephacel column (No. 15 - 30, 96 ml) were concentrated to

5.0 ml and applied to a Sephacryl S-300 column (2.0 x 74 cm). The active fractions (No. 32 - 39, 24 ml) were applied to the Blue-Sepharose CL-6B column after concentrated to 2.1 ml. The fractions exhibiting the enzyme activity (No. 39 - 43, 25 ml) were eluted with 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM ATP and 1 M KCl. These fractions were pooled and used as an acetate-activating enzyme preparation. All these purification steps were carried out at 0 - 4 °C.

The purification procedures for the propionate-activating enzyme are summarized in Table 1. This enzyme preparation was stored at -20 °C because the enzyme was very stable under the conditions of freeze/thaw, while the acetete-activating enzyme was unstable under these conditions.

The final preparation of the propionate-activating enzyme was homogeneous judged from the polyacrylamide gel electrophoresis in the absence of sodium dodecylsulfate, as shown in Fig. 4.

3. Characterization of the purified propionate-activating enzyme

Table 1. Purification of the propionate-activating enzyme from Candida tropicalis

Fraction	Protein (mg)	Total activity (nmol/min)	Yield (%)	Specific activity (nmol/min/mg)	Purification (fold)
Cell homogenate	546	32,600	100	59.7	1
Cell-free extract	321	31,900	98	99.4	1.7
DEAE-Sephacel eluate	20.6	8,050	25	391	6.5
Sepharose 6B eluate	4.93	5,800	18	1,180	20
Blue-Sepharose CL-6B eluate	1.21	4,060	12	3,360	56

Enzyme activity was measured using propionate as substrate

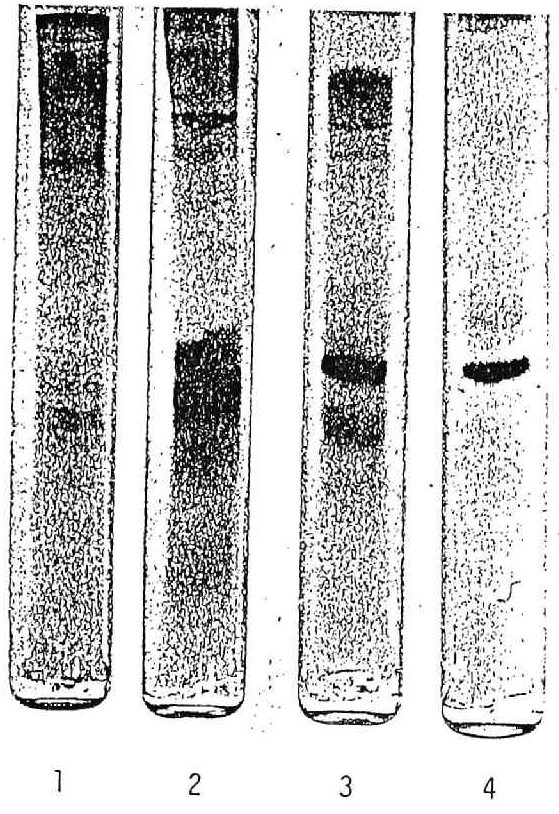


Fig. 4. Polyacrylamide gel electrophoretic patterns of the active fractions obtained at each step of the purification of the propionate-activating enzyme.

Aliquots (13 μg of protein) of the pooled fractions were applied to the gels in the absence of sodium dodecylsulfate. 1, cell-free extract; 2, DEAE-Sephacel eluate; 3, Sepharose 6B eluate; 4, Blue-Sepharose CL-6B eluate (the purified enzyme).

To estimate the molecular mass of the enzyme, polyacrylamide slab-gel electrophoresis (10 % acrylamide) with sodium dodecylsulfate and the gel filtration chromatography were carried out. The subunit of the propionate-activating enzyme was found to have the molecular mass of 87 kDa. On the other hand, the molecular mass of the subunit of the acetate-activating enzyme partially purified from the yeast was 72 kDa. On the gel filtration chromatography, the propionate-activating enzyme was eluted at the position of the molecular mass of about 90 kDa. These results indicate that the propionate-activating enzyme consists of a single peptide with a molecular mass of 87 kDa.

As shown in Table 2, there were many differences in the properties between the purified propionate-activating enzyme and the partially purified acetate-activating enzyme. Although both enzymes were localized in cytosol, the propionate-activating enzyme was synthesized only in the propionate-grown cells, while the acetate-activating enzyme was synthesized in the acetate- and propionate-grown cells. Their behaviours in chromatography on DEAE-Sephacel and Blue-Sepharose CL-6B were also quite different. The propionate-activating enzyme showed an acidic optimal pH

Table 2. Properties of the propionate-activating and acetate-activating enzymes from the propionate-grown cells of Candida tropicalis

Property	Activating Enzyme	
	Propionate-	Acetate-
Localization	cytoplasm	cytoplasm
Biosynthesis	inducible	constitutive
KCl concentration of DEAE-Sephacel elution (M)	> 0.15	< 0.15
Adsorption on Blue-Sepharose CL-6B	-	+
Molecular mass (kDa)		
Native enzyme	90	—[a]
Subunit	87	72
Optimal pH	5.5	7.5
Relative activity (%) on		
Acetate	32	100
Propionate	100	34
Butyrate	50	2
Valerate	26	9
Stability[b]	stable	unstable

[a] Not examined.

[b] Each enzyme was stored at -20 °C and 4 °C for one month.

and more broad substrate specificity than the acetate-activating enzyme. The former showed the highest activity on propionate, while the latter on acetate. There was also a great difference between the stability of the enzymes. The propionate-activating enzyme was much more stable during storage at -20 °C and 4 °C for one month than the acetate-activating enzyme was. These results indicated that the propionate-activating and acetate-activating enzymes were distinct proteins.

As shown in Table 3, the activity of the acetate-activating enzyme was absolutely dependent on ATP, CoA and Mg^{2+}, while that of the propionate-activating enzyme was almost independent of these compounds. Glutathione was not necessary in both reactions. Based on the dependency on ATP and CoA, the acetate-activating enzyme was proved to be typical acetyl-CoA synthetase. On the other hand, the reaction with the propionate-activating enzyme seemed to be very unique, differing from the synthetase-like reaction. Even when the contaminations of ATP, CoA and phosphate ion were avoided carefully during the purification of the enzyme, similar results were obtained.

If the propionate-activating enzyme was a kind of

Table 3. Effects of reaction components on the formation of hydroxamates by the propionate-activating and acetate-activating enzymes

Propionate and acetate were used as substrates for the propionate-activating and acetate-activating enzymes, respectively.

Omission	Relative enzyme activity (%)	
	Propionate-activating enzyme	Acetate-activating enzyme
None	100	100
Fatty acid	0	0
Mg^{2+}	98	0
ATP	81	0
CoA	97	0
ATP and CoA	94	0

propionyl-CoA synthetase, propionyl-CoA must be a reaction intermediate in the assay system employed. However, any derivative of propionate was not detected before the addition of hydroxylamine to the reaction system so far as the product was surveyed by means of thin layer chromatography (Table 4). After the addition of hydroxylamine, the formation of propionylhydroxamate was clearly observed. In the reaction catalyzed by the acetate-activating enzyme, acetyl-CoA and acetylhydroxamate were detected before and after the addition of hydroxylamine, respectively, to the reaction system. These products were also identified by co-chromatography with the authentic samples on a thin layer plate. These facts also revealed the acetate-activating enzyme to be acetyl-CoA synthetase.

The results obtained here indicate that the propionate-activating enzyme is a unique enzyme but not a kind of synthetase, although the activation mechanism catalyzed by this enzyme has not been elucidated yet.

DISCUSSION

Mishina et al. (6,7) reported the presence of two

Table 4. Identification of the reaction intermediates and final products in the propionate-activating and acetate-activating enzyme reactions

Each aliquot of the reaction mixtures before the addition of hydroxylamine (intermediates) and after the addition of hydroxylamine (final products) and authentic samples were chromatographed on a cellulose-coated thin layer sheet using a solvent system of n-butanol/acetic acid/water (4/1/5, v/v/v). After drying the sheet, spots of intermediates, authentic acetyl-CoA and authentic propionyl-CoA were observed under the UV light. Spots of final products, authentic acetylhydroxamate and authentic propionylhydroxamate were visualized by spraying 0.66 M HCl solution containing 10 % trichloroacetic acid and 15 % $FeCl_3.6H_2O$.

Sample	Propionate-activating Enzyme (Substrate:Propionate)	Acetate-activating Enzyme (Substrate:Acetate)
	(Rf value)	
Product before hydroxylamine addition	ND[a]	0.16
Authentic acetyl-CoA	—	0.16
Authentic propionyl-CoA	0.25	—
Product after hydroxylamine addition	0.70	0.55
Authentic acetylhydroxamate	—	0.55
Authentic propionylhydroxamate	0.70	—

[a] Not detectable.

distinct long-chain fatty acyl-CoA synthetases in Candida lipolytica cells grown on alkanes or long-chain fatty acids. Acyl-CoA synthetase I localized in mitochondria and microsomes plays a role in lipid synthesis, while acyl-CoA synthetase II detected exclusively in peroxisomes participates in fatty acid oxidation. These enzymes are distinguishable each other by the immunochemical property and the requirement of phosphatidylcholine. Although existence of such different types of acyl-CoA synthetases have not been recognized in C. tropicalis cells grown on alkanes, the enzyme was also localized in peroxisomes, mitochondria and microsomes, having distinct metabolic functions according to its distinct localization (8). In addition to acyl-CoA synthetase specific for long-chain fatty acids, enzymes active on short-chain fatty acids were found in the cells of C. tropicalis. An enzyme most active on propionate was detected in propionate-grown cells, and another enzyme most active on acetate was found in both acetate-grown and propionate-grown cells. These short-chain fatty acid-activating enzymes were distinguishable by the chromatographic behaviour on DEAE-Sephacel and by the substrate specificity. Both enzymes were exclusively

localized in cytoplasm.

A novel enzyme, the propionate-activating enzyme, was induced in the propionate-grown cells of Candida tropicalis. This enzyme was quite unique, differing from the acetate-activating enzyme (acetyl-CoA synthetase) present in the same cells not only in the molecular size but also in the catalytic properties. That is, acetyl-CoA synthetase formed acetylhydroxamate via acetyl-CoA, whose formation was dependent on ATP and CoA, while the propionate-activating enzyme did not synthesize an intermediate propionyl-CoA. Furthermore, the formation of propionylhydroxamate was independent of ATP and CoA.

Hitherto, acetyl-CoA synthetase has been known to be mainly responsible for the activation of short-chain fatty acids in animals (9,10), a plant (11), a yeast (12) and a bacterium, Rhodospirillum rubrum (13). Propionate is usually discussed as one of the substrates for acetyl-CoA synthetase. However, two reports have been published on propionate-activating enzymes. One enzyme was partially purified from Rhodopseudomonas sphaeroides (14), and another from guinea pig mitochondria (15). These two enzymes are propionyl-CoA synthetase because of requirement

of ATP and CoA for their activities. Therefore, it is clear that the enzyme purified from C. tropicalis is quite different from these synthetases in the reaction mechanism.

A transient activated form of propionate or an activated enzyme-propionate complex etc. may be assumed because of the independency of ATP in its reaction. Until the elucidation of the reaction mechanism, we would like to call this new enzyme as the propionate-activating (propionylhydroxamate-forming) enzyme.

SUMMARY

A novel propionate-activating (propionylhydroxamate-forming) enzyme, which was different from an acetate-activating enzyme (acetyl-CoA synthetase), was found to be induced in a propionate-grown yeast, Candida tropicalis. The enzyme consisted of a single peptide with molecular mass of 87 kDa and exhibited a pH-optimum of 5.5. In addition to propionate, acetate, butyrate and valerate served as substrates. The formation of hydroxamate was independent of ATP and CoA.

In contrast, acetyl-CoA synthetase constitutively

present in the cytoplasm of acetate- and propionate-grown yeast cells had a subunit molecular mass of 72 kDa and a pH-optimum of 7.5. Activation of acetate and propionate by this enzyme required ATP and CoA.

These observations suggest that the novel enzyme has a reaction mechanism different from that of the synthetase.

REFERENCES

(1) Fukui,S. & Tanaka,A. (1979) J. Appl. Biochem. 1, 171.

(2) Fukui,S. & Tanaka,A. (1979) Trends Biochem. Sci. 4, 246.

(3) Tanaka,A., Osumi,M. & Fukui,S. (1982) Ann. N. Y. Acad. Sci. 386, 183.

(4) Ueda,M., Tanaka,A. & Fukui,S. (1982) Eur. J. Biochem. 124, 205.

(5) Ueda,M., Tanaka,A. & Fukui,S. (1984) Eur. J. Biochem. 138, 445.

(6) Mishina,M., Kamiryo,T., Tashiro,S., Hagihara,T., Tanaka,A., Fukui,S., Osumi,M. & Numa,S. (1978) Eur. J. Biochem. 89, 321.

(7) Mishina,M., Kamiryo,T., Tashiro,S. & Numa,S. (1978)

Eur. J. Biochem. 82, 347.

(8) Yamada,T., Nawa,H., Kawamoto,S., Tanaka,A. & Fukui,S. (1980) Arch. Microbiol. 128, 145.

(9) Campagnari,F. & Webster,T.,Jr. (1963) J. Biol. Chem. 238, 1628.

(10) Hele,P. (1954) J. Biol. Chem. 206, 671.

(11) Abraham,A. & Bachhawat,B.K. (1962) Biochim. Biophys. Acta 62, 376.

(12) Berg,P. (1956) J. Biol. Chem. 222, 991.

(13) Eisenberg,M.A. (1955) Biochim. Biophys. Acta 16, 58.

(14) Maruyama,K. (1982) J. Biochim. 91, 725.

(15) Groot,P.H.E. (1976) Biochim. Biophys. Acta 441, 260.

GENERAL CONCLUSION

The present study has been performed to elucidate the physiological significance of peroxisomes in the alkane assimilation pathway, especially, the metabolism of fatty acids derived from alkanes in Candida tropicalis.

The enzymes related to the fatty acid β-oxidation ----- acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase ----- were induced in C. tropicalis cells assimilating alkanes. These enzymes were localized only in peroxisomes, while none of these enzymes nor acyl-CoA dehydrogenase, which is known to participate in the initial step of the mitochondrial β-oxidation in mammalian cells, were detected in yeast mitochondria under the conditions employed.

Activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were involved in a bifunctional protein of a single peptide in peroxisomal fatty acid β-oxidation system of C. tropicalis. The author purified a bifunctional enzyme with a molecular mass of 105 kDa having enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities and the enzyme with four subunits of a molecular mass of 36 kDa

having only enoyl-CoA hydratase activity. The latter was clarified to be derived from a bifunctional enzyme by the action of a certain protease in the yeast cells.

Acetyl-CoA formed in the fatty acid β-oxidation enters into the glyoxylate cycle which includes isocitrate lyase and malate synthase as key enzymes. Isocitrate lyase and malate synthase were induced in n-alkane-, acetate-, and propionate-grown cells, and were localized predominantly in mature peroxisomes of alkane-grown cells and in pre-existing peroxisomes of glucose-grown cells, while the enzymes were localized predominantly in cytoplasm in propionate-grown cells having immature peroxisomes, and both in pre-existing peroxisomes and cytosol of acetate-grown cells. Both enzymes were purified from the peroxisomes of alkane-grown cells, and compared with the enzymes purified from glucose-, acetate- and propionate-grown cells. Isocitrate lyase isolated from the respective sources had a molecular mass of 130 kDa, composed of two identical subunits. All malate synthase had a molecular mass of 250 kDa, composed of four identical subunits. There were little differences in kinetic, proteinaceous and immunochemical properties in the respective enzymes purified from yeast cells grown on the

different carbon sources, the results indicating that the different forms of the active and inactive enzymes were not present in the yeast cells. The author expects these facts to be a clue to investigate the peroxisome biogenesis and transport mechanism of peroxisomal proteins.

In the course of these studies, the author discovered a novel propionate-activating (propionylhydroxamate-forming) enzyme. The enzyme was induced in cytoplasm of propionate-grown C. tropicalis cells. The molecular mass, optimum pH, substrate specificity, and the reaction mechanism of this enzyme were different from those of acetyl-CoA synthetase.

PUBLICATION LIST

1) Peroxisomal localization of enzymes related to fatty acid β-oxidation in an n-alkane-grown yeast, Candida tropicalis

Ueda, M., Yamanoi, K., Morikawa, T., Okada, H. & Tanaka, A.

Agric. Biol. Chem. (1985) 49, 1821-1828.

2) Enoyl-CoA hydratase in a peroxisomal bifunctional enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, from an n-alkane-utilizing yeast Candida tropicalis

Ueda, M., Morikawa, T., Okada, H. & Tanaka, A.

in preparation

3) Properties of isocitrate lyase from an alkane-utilizable yeast, Candida tropicalis

Uchida, M., Ueda, M., Matsuki, T., Okada, H., Tanaka, A. & Fukui, S.

Agric. Biol. Chem. (1986) 50, 127-134.

4) Purification of peroxisomal malate synthase from alkane-grown Candida tropicalis and some properties of the purified enzyme
Okada, H., Ueda, M. & Tanaka, A.
Arch. Microbiol. (1986) 144, 137-141.

5) Comparison and subcellular distribution of isocitrate lyase and malate synthase in Candida tropicalis cells grown on different carbon sources
Okada, H., Ueda, M., Uchida, M. & Tanaka, A.
in preparation

6) Purification of a novel propionate-activating (propionyl-hydroxamate-forming) enzyme from propionate-grown cells of Candida tropicalis
Ueda, M., Okada, H., Tanaka, A. & Fukui, S.
Agric. Biol. Chem. (1985) 49, 3613-3614.